

Original Article

Respiratory disease in Niemann-Pick type C2 is caused by pulmonary alveolar proteinosis

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Niemann-Pick diseases are hereditary neurovisceral lysosomal lipid storage disorders, of which the rare type C2 almost uniformly presents with respiratory distress in early infancy. In the patient presented here, the NPC2 exon 4 frameshift mutation c.408_409delAA caused reduced NPC2 protein levels in serum and lung lavage fluid and the synthesis of an aberrant, larger sized protein of around 28 kDa. Protein expression was strongly reduced also in alveolar macrophages. The infant developed failure to thrive and tachypnea. Lung lavage, computer tomography, and histology showed typical signs of pulmonary alveolar proteinosis with an abnormal intraalveolar accumulation of surfactant as well as macrophages. An NPC2-hypomorph animal model also showed pulmonary alveolar proteinosis and accumulation of macrophages in the lung, liver, and spleen long before the mice died. Due to the elevation of cholesterol, the surfactant had an abnormal composition and function. Despite the removal of large amounts of surfactant from the lungs by therapeutic lung lavages, this treatment was only temporarily successful and the infant died of respiratory failure. Our data indicate that respiratory distress in NPC2 disease is associated with a loss of normal NPC2 protein expression in alveolar macrophages and the accumulation of functionally inactive surfactant rich in cholesterol.

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Niemann-Pick diseases represent a family of neurovisceral lysosomal lipid storage disorders, many of which lead to severe neurodegeneration and early death. Types A (OMIM #257200) and B (OMIM #607616) result from an inherited deficiency of lysosomal acid sphingomyelinase activity. Niemann-Pick type C1 disease (OMIM #257220) is caused by mutations in the *NPC1* gene (1), which codes for the NPC1 protein. This is an integral membrane protein that is primarily localised to late endosomes/lysosomes and also cycles through the Golgi apparatus (2, 3). Niemann-Pick type C2 disease (OMIM #607625) is caused by mutations in the *NPC2* gene (4). It encodes the NPC2 protein, in its mature form a 132 amino-acid, soluble, ubiquitously expressed, mannose 6-phosphorylated lysosomal glycoprotein with highest levels in testes, kidney, and liver. NPC2 protein binds unesterified cholesterol with high affinity (5, 6) and transports it to phospholipid vesicles (7, 8). Patients with NPC2 mutations represent the smallest group of Niemann-Pick patients. Up to now, 27 different families with a defined mutational status have been described (9–12). In contrast to the other types of Niemann-Pick disorders, many of these patients present with respiratory distress in their early infancy (9). There are only 18 infantile cases with genetically proven NPC2 disease. Information on respiratory symptoms within the first six months of life was only available in 14 patients, all of which had developed significant symptoms. The median age of death from respiratory insufficiency was 8 months (range 2.5–54). The pathophysiological mechanisms of respiratory distress in Niemann-Pick type C2 disease are still unclear.

In a recent report of two infants with Niemann-Pick disease type C2, recurrent bronchitis and bronchiolitis with respiratory failure were suspected (13). Histopathology of autopsy lungs documented increasing pulmonary fibrosis, progressive lung interstitial involvement, and lipid pneumonia (9, 14–16), whereas in biopsy specimens a histological pattern of pulmonary alveolar proteinosis predominated (17, 18).

Alveolar proteinosis is a rare pulmonary disease characterised by an intraalveolar accumulation of surfactant. Pulmonary surfactant is a complex mixture of proteins and lipids that normally covers the alveolar surface to reduce surface tension (19). Until now, four surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) have been characterised. Dipalmitoyl phosphatidylcholine, phosphatidylglycerol, and cholesterol are the principal lipid components. The most frequent form of alveolar proteinosis is caused by autoantibodies against

granulocyte-macrophage colony-stimulating factor (GM-CSF), which is a haematologic growth factor regulating the uptake of surfactant by alveolar macrophages (20). In cases of primary alveolar proteinosis in infants, mutations of the surfactant proteins B or C or of the alpha chain of the GM-CSF receptor were found (21–23). Secondary pulmonary alveolar proteinosis develops in association with inhalation of inorganic dusts like silica or titanium, some forms of leukaemia, and certain infections (20). The role of alveolar proteinosis in the pathophysiology of respiratory distress in Niemann-Pick disease type C2, however, needs to be clarified.

Case, materials and methods

Case report

The parents of the patient were unrelated, the pregnancy was normal, and the female child was born at term. She had a 5 year old sister in good health. The mother noticed progressive tachypnea, failure to thrive, and poor feeding since the infant's second month of life. Within four months, she was admitted to the Hospital Infantil de México, Mexico City, Mexico with dyspnea, peripheral cyanosis, fever, and non-productive cough. She was diagnosed with bronchiolitis and cardiac failure. The initial chest X-ray (Fig. 1c) showed bilateral micronodular infiltrates particularly in the lower left lung and apical right lobe. The lungs were overinflated, and basal fine crackles were noticed. Liver and spleen were enlarged to 4 and 5 cm, respectively. Clubbing of the fingers was present. Neurology and neurological development were normal.

Pneumonia was then diagnosed and she was treated with Cefuroxime and Claritromycin. Microbiologic investigations, a sweat test, immunoglobulins as well as blood and urine metabolic screening were normal. Serum triglycerides were moderately elevated [380 mg/dl (normal <150)], while the cholesterol concentration was normal [169 mg/dl (normal <170)] with a slight elevation in LDL cholesterol [115 mg/dl (normal <110)] and a slight decrease in HDL cholesterol [31 mg/dl (normal >35)]. The abdominal CT scan showed hepatosplenomegaly. Chest CT scan and bronchoalveolar lavage (BAL) pointed towards a diagnosis of alveolar proteinosis. Open liver, spleen, and lung biopsies were performed, and the diagnosis of Niemann-Pick type C disease and pulmonary alveolar proteinosis was made.

In an attempt to treat the deteriorating respiratory function with the prominent feature of alveolar

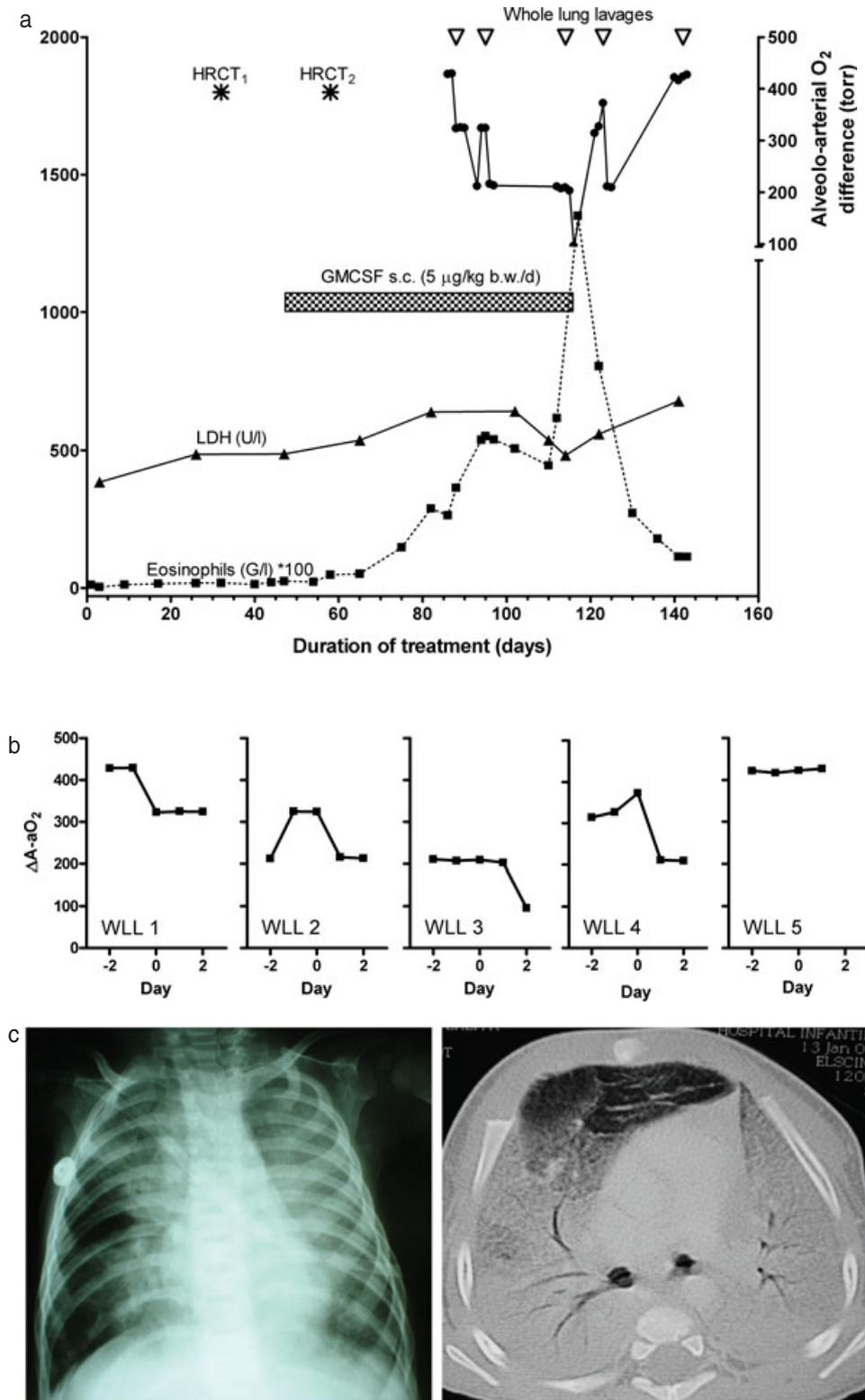


Fig. 1. Clinical course and characteristics of the patient. (a) no clinical effect of s.c. GM-CSF on the respiratory situation, indicated by increasing levels of LDH and continuous deterioration of the respiratory status. Note the intact response to GM-CSF as indicated by the developing massive eosinophilia. Whole lung lavages initially led to a consistent but only transient improvement of gas exchange (a,b). The 1st half lung lavage (HLL) was done on the right side, the 2nd on the left side 7 days after the 1st, the 3rd (right lung) 19 days later, the 4th (left lung) 9 days later and the last (right lung) 20 days later. (c) the chest X-ray on admission and the first CT scan (* CT₁ in a).

proteinosis, subcutaneous treatment with GM-CSF was initiated, but without clinical effect. Standard treatment with half lung lavages was then introduced to treat the pulmonary alveolar proteinosis. This was initially successful (Fig. 1), but after the fifth lavage the patient developed a pneumothorax. She deteriorated rapidly and died the following day. The parents did not consent to perform a post-mortem examination.

Half lung lavages

Under general anesthesia and endotracheal intubation, a 3.5 mm diameter fiberoptic bronchoscope was inserted via a tracheostomy. With the airway safe, the fiberoptic bronchoscope was wedged in the lobar bronchi of one side of the lungs for each half lung lavage. Each lobe was washed with 15-ml aliquots of pre-warmed isotonic saline solution until a clear aspirate was obtained. During the procedure, positional and assisted clearance using manual chest physiotherapy was performed.

Samples from control or comparison subjects

Serum and lavage fluid was used from both an adult patient with autoimmune alveolar proteinosis caused by antibodies against GM-CSF and the patient with congenital SP-C deficiency due to the SFTPC I73T mutation (21). In addition, normal control sera from lab personnel were obtained. To assess surface tension, natural bovine surfactant was used as control in *in vitro* experiments (24).

Preparation of lavage and surfactant fractions

To sediment the cells, lavage fluid was centrifuged at 200 g for 10 min. The supernatant was either used directly for Western blotting and biochemical analyses or further centrifuged at 40,000 × g for 30 min in order to separate the surfactant into the large surfactant aggregate fraction (pellet) and the small surfactant aggregates (supernatant) to perform surface tension measurements using the pulsating bubble surfactometer (24).

Mutation analysis and biochemical assays

Genomic DNA was isolated from whole blood and the genes of Niemann-Pick diseases (*SMPD1*, *NPC1*, *NPC2*) and of the pulmonary surfactant-associated proteins B and C (*SFTPB* and *SFTPC*) were sequenced following standard protocols (25).

Acid sphingomyelinase activity in peripheral leukocytes of the patient was normal (3.9 nmol/mg per hour; normal controls 3.3–6.4). The level of

GM-CSF autoantibodies in serum was determined as described previously (26). For Western blotting, proteins were separated under reducing conditions [NuPage10% Bis-Tris gels (Novex, San Diego, CA)] and incubated with antibodies against pro-SP-B, SP-C, SP-B (27), or NPC2 (28). ELISAs for concentrations of surfactant proteins (SP) A and D were performed as described, allowing for minor modifications (24). Lavage or homogenized cells were extracted, and lipid classes and subspecies were determined by electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis (29).

Analysis of surfactant activity in the pulsating bubble surfactometer

Surface tension of surfactant isolated from lavages was determined at phospholipid concentrations of 1 and 3 mg/ml (24). Inhibition of surface activity by cholesterol (Sigma, Taufkirchen, Germany) or ceramide [(*N*-Nervonoyl-*D*-erythro-Sphingosine (C24:1 Ceramide), Avanti, Polar lipids, Alabaster, AL] was tested after admixture of the lipids to lyophilized large aggregate surfactant and reconstitution in bubble buffer [140 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*-ethane sulfonic acid (HEPES), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 3.5 mM CaCl₂, pH 6.9].

Histology and immunohistochemistry of biopsy material and of NPC2-deficient mice

Lung biopsy specimens were fixed in 4% buffered formaldehyde and immunostained using alkaline phosphatase (30). Tissues from female NPC2-deficient mice of mixed genetic background (129/C57B16/BALBC, Nr 9645) and littermate controls (Nr 9644) were provided by the Lobel laboratory (31). Day 41 animals were analysed. At this point of time, mortality was zero, the natural life cycle of such NPC2 deficient mice being premature death. The first mice died aged 56 days. The median survival for this genotype is 110 days. The control animals were healthy and developed no symptoms. The tissue of two animals from each group was assessed by a pathologist blinded to the group assignment.

Ethics

Written informed consent was obtained from all subjects involved. The procedures during treatment and the usage of case data and materials were approved by the ethics review boards of the participating institutions.

Results

Novel homozygous Niemann-Pick C2 mutation c.408_409delAA in an infant with chronic respiratory distress

By light microscopy, large macrophages with a foamy cytoplasm (*arrows*) were found within the liver (Fig. 2a) and spleen (Fig. 2d). Since foamy macrophages are easily overlooked in the liver due

to increased vacuolation and decreased eosinophilia of hepatocytes (*arrowheads*), Niemann-Pick type C was suggested. Proof of lipid inclusions consisting of concentrically laminated myelin-like figures within foamy macrophages at the ultrastructural level was confirmatory (Fig. 2a,d, insets). Sequencing of the *NPC2* gene demonstrated the homozygous deletion of two adenosines at amino acid position 136/137 in exon 4 (c.408_409delAA)

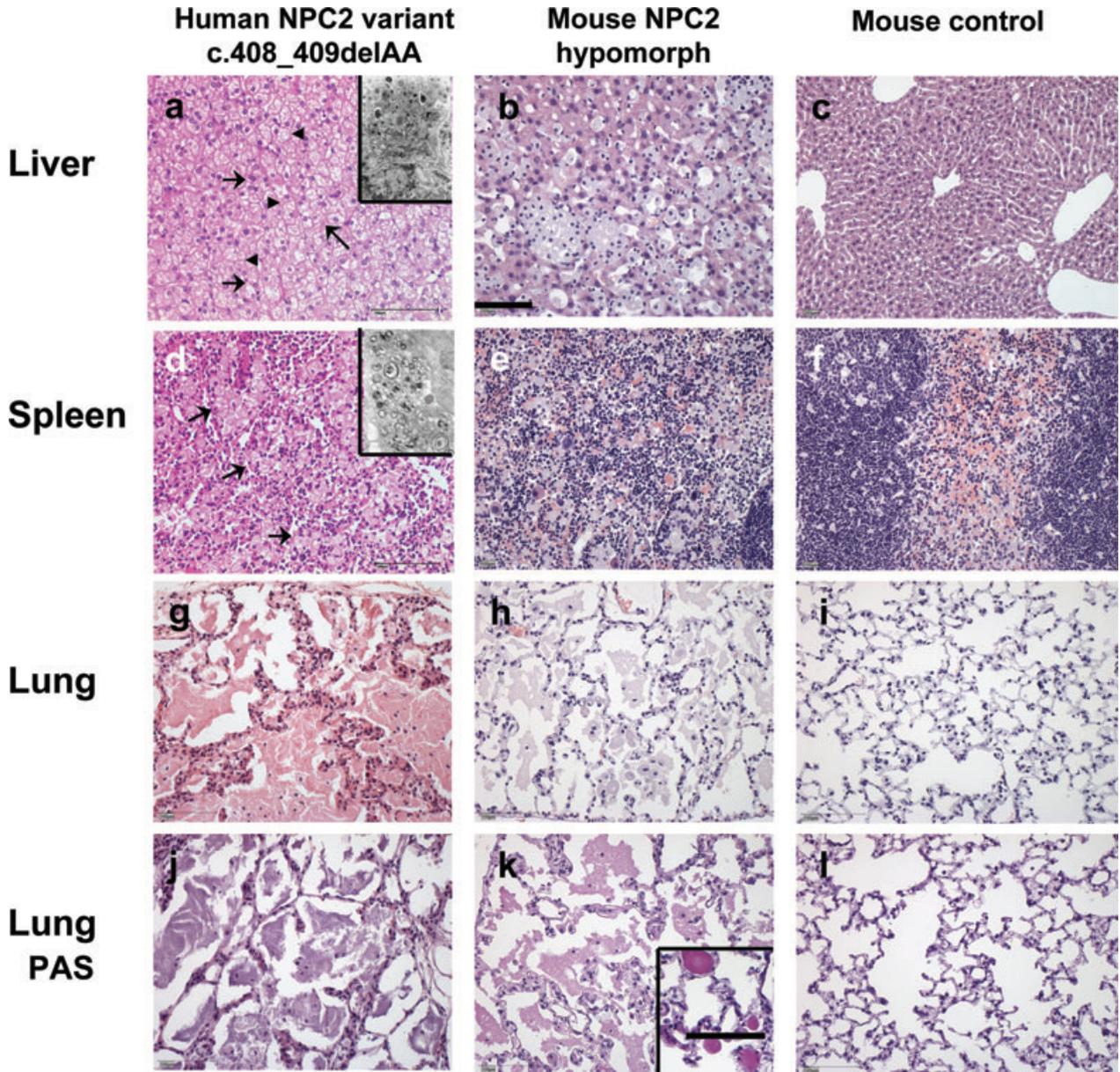


Fig. 2. Comparison of human (left panels **a, d, g, j**) and mouse [middle (**b, e, h, k**) and right panels (**c, f, i, l**)] histology of liver, spleen, and lung (panels **a** to **i** all HE stain, bar 100 µm) from an open lung biopsy of the infant with Niemann-Pick type C2 disease, from the NPC2-hypomorph animal model of Niemann-Pick type C2 disease (on day 41), and from the corresponding control mice. The inserted electron micrographs demonstrate the typical histology of Niemann-Pick C disease (panels **a, d** with insets each). The lower three panels (**j, k, l**) show lung tissue stained with the typical findings of pulmonary alveolar proteinosis and lung tissue of a healthy control mouse. The inset in panel **k** (bar 50 µm) illustrates oval bodies' characteristic of pulmonary alveolar proteinosis.

which results in a reading frame shift, the insertion of 93 new amino acids, and the synthesis of a longer, immature NPC2 protein with 231 instead of 151 residues (Fig. 3a).

Reduced expression of normal NPC2 protein in lavage, serum, and alveolar macrophages

The amount of NPC2 in bronchoalveolar lavage fluid of the patient was reduced to less than 1/100 of the amount of comparison samples (Fig. 3c). This loss was mainly due to the absence (serum) or reduction (lavage) of the typical doublet of

NPC2 running in our system at about 19 kDa. In addition a novel band was observed at a molecular weight of 28 kDa, indicating the presence of the elongated protein in lavage (lanes 2 and 3, Fig. 3c) and serum (lane 7, Fig. 3c). The band at 19 kDa detected in the patient only in lavage may represent a degradation product from the larger 28 kDa protein or a cross-reacting band in lavage that is normally covered by the much stronger 19 kDa NPC2 immunoreactivity.

The reduced NPC2 levels were confirmed by immunohistological staining of lung tissue. In normal control tissue, alveolar macrophages were

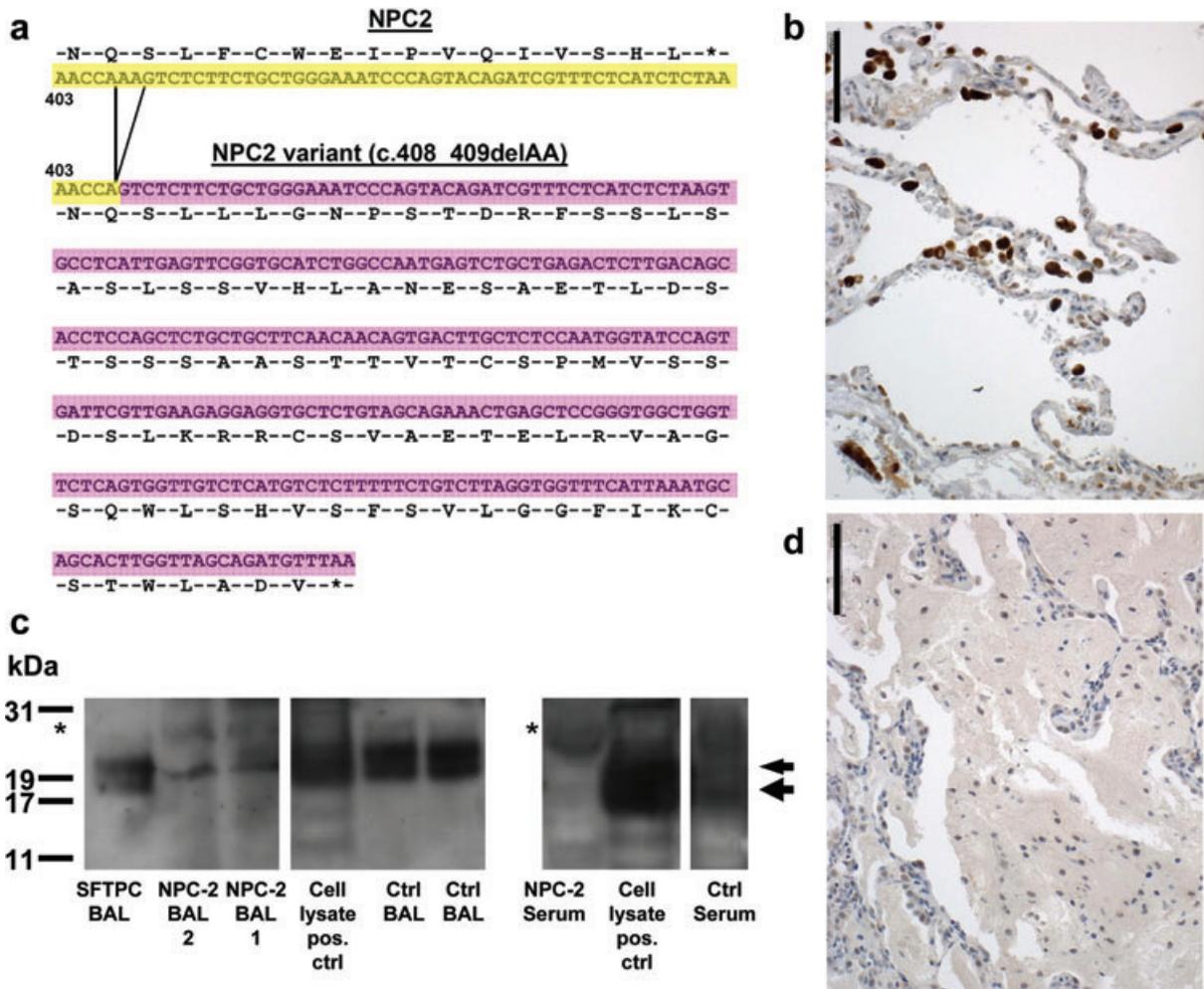


Fig. 3. Panel (a) depicts the C-terminal sequence of the normal NPC2 gene and NPC2 protein from nucleotide 403 onward (yellow) and that of the mutated NPC2 variant after deletion of the two nucleotides at positions 408 and 409 in exon 4, resulting in a reading frame shift and a longer protein product (pink). Panel (c) shows Western blots of lavage samples (BAL, 5 µg were applied to lane 1, 25 and 35 µg to lanes 2 and 3 and 10 µg to lanes 5 and 6) from a control patient with alveolar proteinosis due to the surfactant protein C mutation I73T, two lavages from the case with the NPC2 mutation c.408_409delAA (NPC-2), and BAL from healthy subjects (Ctrl). Cell lysates (40 µg of protein) from macrophages of normal subjects served as a positive control (arrows). Serum samples (20 µg per lane) from the NPC2 patient and healthy controls are depicted on the right hand of panel (c). Note that in the patient's serum typical NPC2-immunoreactive bands were lacking but that an aberrant band was present at about 28 kDa (asterisk). In BAL, the aberrant 28 kDa band was reactive in addition to a faint band at 19 kDa. Immunohistochemical staining of NPC-2 protein in a healthy control (note brown staining of macrophages) and in the patient with the NPC2 mutation is shown in Panels (b) and (d). Note the missing brown NPC2-stain in the alveolar macrophages.

primarily stained (Fig. 3b,d), as confirmed by colocalization of the staining pattern with the CD68 stain (macrophages), but not with the MNF116 stain, which stains epithelial cells (not shown).

The chronic respiratory distress is due to pulmonary alveolar proteinosis

The characteristic clinical course with the insidious development of dyspnoea exaggerated by a respiratory tract infection with fever, the initial diagnostic BAL with minimal inflammatory cells, no cultural growth except for a few colonies of *Stenotrophomonas maltophilia*, opaque and milky fluid lavage fluid with plenty of oval bodies and eosinophilic amorphous periodic acid-Schiff (PAS)-positive material microscopically (not shown), and enlarged foamy and vacuolated alveolar macrophages, and the CT scan were typical of a pulmonary alveolar proteinosis (Fig. 1c).

Open lung biopsy showed histological patterns typical of pulmonary alveolar proteinosis on one hand (Fig. 2, panels g, j) and typical Niemann-Pick disease on the other hand (Fig. 2, panels a, d, g). The alveolar spaces were filled with a granular, eosinophilic material (Fig. 2, panel g) that was PAS stain-positive (Fig. 2, panel j) and diastase-resistant (not shown). Furthermore, a hyperplasia of type II pneumocytes and intraalveolar as well as interstitial accumulation of foamy macrophages were present.

Special stains for the pulmonary surfactant proteins characterised the surfactant metabolism. Intraalveolar accumulation of SP-A, which is

pathognomonic for alveolar proteinosis (Fig. 4, panel b), was confirmed by biochemical analysis of lavage fluid for SP-A, and extended to SP-D (Table 1). Precursors of SP-B and SP-B were also very abundant in the alveolar space (Fig. 4, panels c, d), whereas precursors of SP-C were restricted to type II pneumocytes (Fig. 4, panels e and f). SP-C was abundantly present in the alveolar space, both with normal molecular weight (about 4.7 and 10 kDa) and with higher molecular weight forms which are typical for alveolar proteinosis (Fig. 4, panel f).

Alveolar surfactant is abnormal in lipid species composition and surfactant proteins

The lipid composition of the alveolar surfactant material recovered was significantly different from that of a normal alveolar surfactant (Table 1). The percentage of the surface-active lipids phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol was reduced, whereas cholesterol, glucosylceramide, ceramide, and sphingomyelin were increased several fold (Table 1).

Interestingly, alterations of the fatty acid composition of the lipids were minor. Representative for the large number of fatty acids investigated, the results for the most important and abundant palmitoyl species are given (Table 2). Dipalmitoylphosphatidylcholine, the major surfactant phospholipid, was present in the same abundance as in controls (Table 2). In addition to the huge increase in SP-A, the increase of cholesterol to about 50% of total surfactant lipid mass and the more than 70-fold increase of ceramide were the most striking

Table 1. Amount and composition of bronchoalveolar lavage fluid (BAL) recovered during therapeutic lavages

	Lavage 1	Lavage 2	Mean fold normal BAL level	Normal level in BAL ^a
Total lipids (nmol/ml)	911.8	899.6	22.2	40.8 ± 9.6
Lipid species (% of total)				
Phosphatidylcholine	33.6	35.2	0.6	55.4 ± 2.1
Sphingomyelin	3.8	3.8	2.9	1.3 ± 0.2
Dihydro sphingomyelin	0.4	0.3	2.4	0.15 ± 0.05
Phosphatidylethanolamine	1.0	0.9	0.2	4.5 ± 0.3
Plasmalogenes	1.6	1.3	2.2	0.7 ± 0.04
Phosphatidylserine	1.9	1.8	0.3	7.0 ± 0.8
Phosphatidylglycerol	0.5	0.8	0.2	3.1 ± 0.08
Phosphatidylinositol	4.6	4.4	0.3	13.2 ± 0.8
Lysophosphatidylcholine	0.2	0.3	0.2	1.5 ± 0.2
Ceramide	0.9	1.2	16.4	0.06 ± 0.01
Glucosylceramide	0.6	0.7	72.2	0.01 ± 0.004
Cholesterol ester	0.9	0.9	0.1	6.2 ± 0.6
Free cholesterol	49.9	48.4	7.0	7.0 ± 0.6
Surfactant protein A (µg/ml)	345	477	79.0	5.2 ± 0.8
Surfactant protein D (ng/ml)	225	294	6.5	80 ± 10

^aData from BALs of healthy children (27)

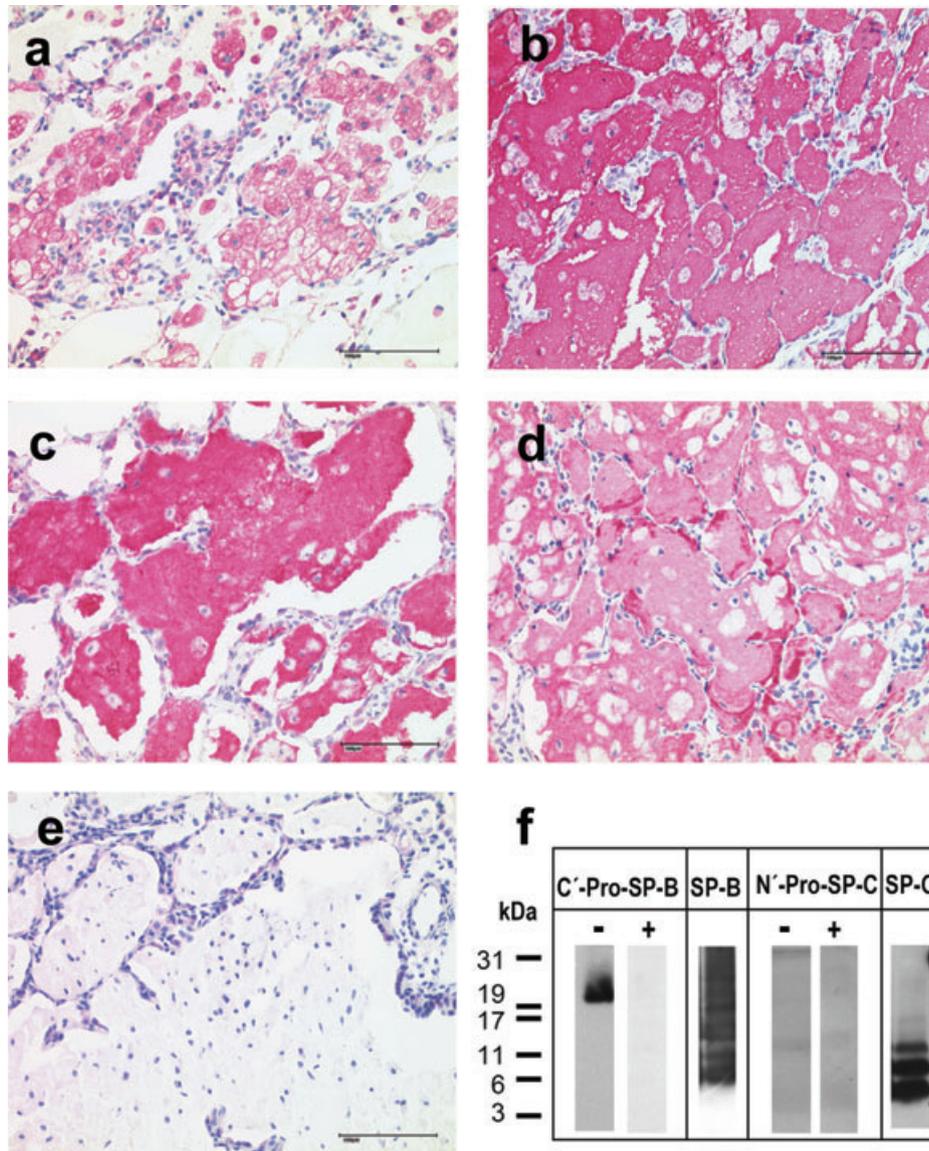


Fig. 4. Immunohistochemical studies of the lung biopsy from the Niemann-Pick type C2 disease patient stained for CD68 (marker of alveolar macrophages)(panel **a**), surfactant protein (SP) A (panel **b**), pro-SP-B and SP-B (panels **c** and **d**), and pro-SP-C (panel **e**; all bars indicate 100 μm). Negative controls and positive immunohistological stains in humans with and without alveolar proteinosis can be found in our previous publication (42). Panel (**f**) shows representative Western blots of BAL from the NPC2 patient. C'pro-SP-B and N'pro-SP-C were probed in the presence (+) and absence (-) of competing peptide to prove the specificity of the bands detected. Immunohistologically identified proteins were confirmed with Western blotting by demonstrating the correct molecular weights.

abnormalities of the patient's alveolar surfactant composition.

Abundant intraalveolar cholesterol likely resulting from NPC2 protein deficiency is the cause of poor surfactant function

Based on the composition data, we hypothesised that the biophysical function of the surfactant recovered from the half-lung lavages of the patient might be very poor (Fig. 5, upper panel). This poor function was not due to the abundance of

proteinaceous inhibitors, as the lipid extract, which contains only the lipid fraction of surfactant, also had a poor function (Fig. 5, middle panel). Similarly, the large increase in surfactant proteins (Tables 1 and 3) did not compensate for dysfunction. However, cholesterol when added to a well-functioning natural surfactant, imitated the loss of function if representing more than 25% of the total lipids (Fig. 5, upper and lower panel), whereas ceramide did not have such a deleterious effect alone nor did it potentiate the cholesterol effect.

Table 2. Fatty acid composition of lipid species with regard to the presence of one palmitoyl (^a16:0) or two palmitoyl (^b32:0) groups

Lipid species	Lavage 1 % of all fatty acids in the species)	Normal level in BAL ^c (palmitoyl, expressed as % of all fatty acids in the species)
Phosphatidylcholine ^b	53	47 ± 4
Sphingomyelin ^a	45	36 ± 9
Dihydrosphingomyelin ^a	6.5	0 ± 0
Phosphatidylethanolamine ^b	0	0
Plasmalogenes ^b	0.3	0.5 ± 0.6
Phosphatidylserine ^b	1.2	0.3 ± 0.7
Phosphatidylglycerol ^b	0	4.2 ± 1.4
Phosphatidylinositol ^b	0	0
Lysophosphatidylcholine ^a	87	40 ± 6
Ceramide ^a	34	56 ± 13
Glucosylceramide ^a	29	6.9 ± 4.3
Cholesterol ester ^a	16	3.7 ± 1.4

^aThe presence of one palmitoyl (16:0) groups.

^bThe presence of two palmitoyl (32:0) groups.

^cNormal lavage data are the mean ± SD from 15 children with chronic bronchitis (27).

Alveolar overfilling with dysfunctional surfactant is responsible for early respiratory symptoms

Initially, when the diagnosis of pulmonary alveolar proteinosis was made, the pathophysiological mechanisms were not entirely clear. Based on reports of successful treatment with GM-CSF (32, 33), a therapeutic trial with subcutaneous GM-CSF was undertaken. Although the infant responded with an appropriate increase in eosinophils (34) (Fig. 1), the clinical and radiological condition as well as the blood levels of lactate dehydrogenase deteriorated further, demonstrating that under these conditions alveolar surfactant homeostasis could not be improved by GM-CSF alone (Fig. 1a).

Half lung lavages removed substantial amounts of lipids and surfactant proteins characteristic of pulmonary alveolar proteinosis from the lungs of this infant (Tables 1–3). With time and disease severity progressing, increasing amounts of total protein, a surrogate for the extent of alveolar proteinosis load, were recovered by the lavages (Table 3). After the first 4 lavages had been performed, there was some improvement in gas exchange (Fig. 1 upper and middle panel). However, after the fifth half lung lavage, the patient developed a pneumothorax and became critically ill, and it was decided together with the parents to withdraw active therapy.

NPC2-hypomorph animal model

In order to substantiate our hypothesis of the development of an alveolar proteinosis in NPC2 disease,

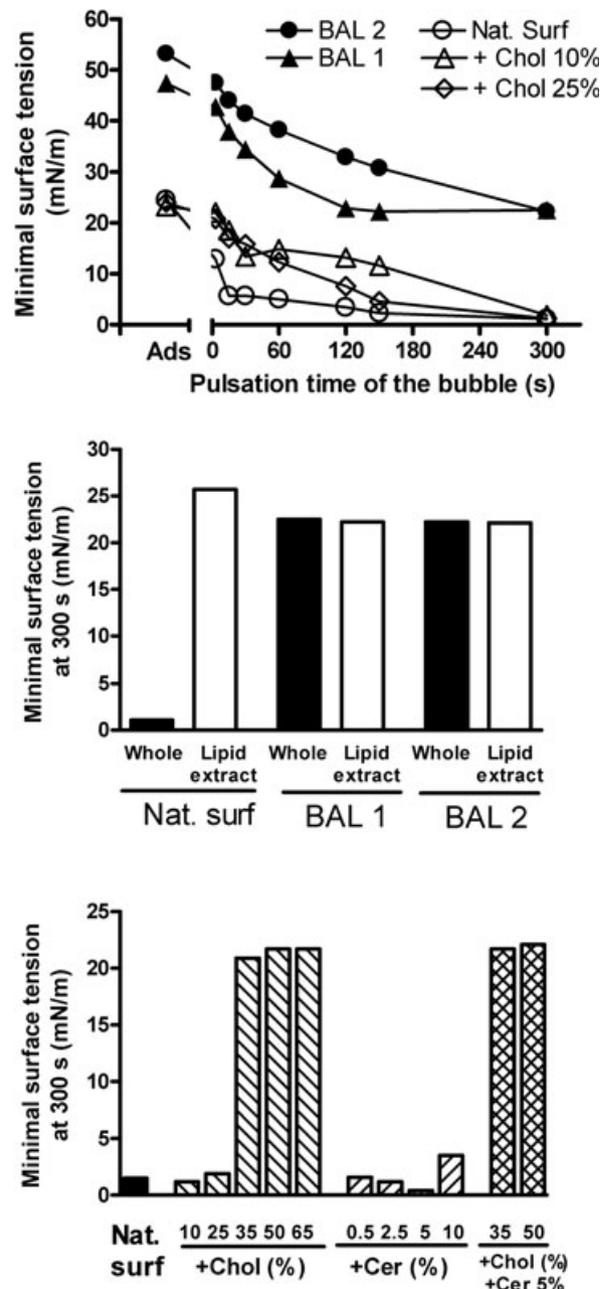


Fig. 5. Surface activity of the patient's bronchoalveolar lavage surfactant, isolated as the large surfactant aggregate fraction (BAL 1 and BAL 2), and of natural surfactant isolated from bovine lung washes (Nat. Surf) were assessed in a pulsating bubble surfactometer at a concentration of 3 mg phospholipid/ml. The surface activity of the surfactant isolated from the NPC2 patient was poor (upper panel). Nat. Surf, in contrast, had an excellent surface activity which was inhibited by cholesterol at increasing concentrations (upper and lower panel) and by the extraction of the hydrophilic surfactant components (lipid extract, middle panel), but not by the addition of ceramide (cer).

lungs of a NPC2-hypomorph animal model, an established model of Niemann-Pick type C2 disease, and healthy littermate control mice were

Table 3. Half lung lavages (warmed 0.9 % NaCl in 15-ml aliquots distributed in all three lobes of the right lung and two lobes of the left lung)

Lavage	Date	Side of segmental lavage	IN (ml)	OUT (ml)	Number of aliquots	Duration (min)	Protein (µg/ml)	Protein removed (mg)	Surfactant protein B (µg/ml)	SP-B removed (mg)	Surfactant protein C (µg/ml)	SP-C removed (mg)
1	10/03/2005	right	650	610	43	70	446	272	n.d.	n.d.	n.d.	n.d.
2	17/03/2005	left	600	540	40	60	619	334	n.d.	n.d.	n.d.	n.d.
3	05/04/2005	right	690	670	46	90	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	14/04/2005	left	630	600	42	70	1801	1081	14.4	8.6	4.4	2.6
5	03/05/2005	right	630	610	42	60	1279	780	20.1	12.1	4.5	2.8

n.a., not available; n.d., not done.

investigated early in the natural course of the disease, i.e. on day 41, when none of the animals had died. In contrast to control mice, NPC2-deficient mice showed an intraalveolar and interstitial accumulation of foamy macrophages and a focal intraalveolar accumulation of a finely granular, PAS-positive material as well as multiple PAS-positive globules typical of alveolar proteinosis (Fig. 2, panels h and k). These data clearly demonstrated that pulmonary alveolar proteinosis is also present at an early point of time in NPC2-deficient mice.

Discussion

An early diagnosis of Niemann-Pick C2 disease is difficult to make as it is not widely known that this disorder usually presents with respiratory symptoms mimicking bronchitis or pneumonia with associated failure to thrive (9, 10, 14, 15). Furthermore, neurological symptoms are less prominent or may not have developed yet, as happened in this case. The novel 2 nucleotide deletion mutation described here results in an elongated NPC2 protein due to a frameshift at amino acid position 136 which generated a new stop codon at residue 232 instead of 152. This longer protein with a molecular weight of about 28 kDa was detected in serum and lavage fluid, but with significantly reduced abundance. Similarly, in the alveolar macrophages, a very low level of NPC2 immunoreactivity was present. The cause for the low levels may be instability of the mutated NPC2 protein with a reduced half-life time intracellularly and in circulation or an increased degradation due to intracellular quality control mechanisms. The normally very high abundance of NPC2 in the lung and, in particular, in alveolar macrophages as demonstrated here, on the other hand, might suggest an important role of this protein in alveolar macrophage effector function, host defence, and metabolism.

In our infant with respiratory distress and Niemann-Pick type C2 disease, we confirmed

the diagnosis of pulmonary alveolar proteinosis by bronchoalveolar lavage, histology, immunohistochemistry, and biochemical analysis of the surfactant. The detection of pulmonary alveolar proteinosis in the NPC2-hypomorph animal model long before the mice died suggests that pulmonary alveolar proteinosis may not be an incidental finding, but may be a common and important feature of early Niemann-Pick type C2. Thus, pulmonary alveolar proteinosis is likely the pathophysiology underlying the initial, early, and unique respiratory symptoms often suggestive of bronchitis or pneumonia. The few histologic reports of patients focussing on this issue are also compatible with the presence of pulmonary alveolar proteinosis during an early disease period (15, 17, 18), whereas at autopsy these findings were absent or less prominent (15–17). At the time of death, which is almost universally caused by respiratory failure, the lungs may in addition show fibrotic changes and an accumulation of foamy macrophages within the interstitial space described as lipid pneumonia (16). This condition is characterised by the accumulation of foamy macrophages and often associated with cholesterol clefts in both the alveolar and interstitial space (35). These data indicate that the histological pattern changes during disease progression. This is in accordance with our finding of a severe lack of NPC2 in alveolar macrophages, which cannot mobilise the cholesterol taken up and thus become foamy macrophages. Since we did not find much NPC2 and cholesterol accumulating in the interstitial space, the disease processes may be much slower there.

The other major finding of this study is a loss of function of the surfactant recovered from the alveolar space. Irrespective of the mechanisms that led to the alveolar proteinosis, the result was a surfactant with defective biophysical activity. This is an uncommon finding, as abundant, but active surfactant is usually recovered in this condition (36). The impaired function was not related to an abundance of inhibitory proteins, as separation by lipid

extraction did not improve the surface activity of the lavages. The extremely high content of SP-A in the patient's surfactant even may have provided some resistance to its biophysical inhibition. We found that the increased content of cholesterol in the surfactant contributed to its impaired surface activity, whereas ceramide was not inhibitory at and above the concentrations found in lavages of the patient. These results are in agreement with earlier studies that demonstrated *in vitro* inhibition of surfactant function by cholesterol (37). In the patient, the cholesterol content was as high as 50% of total surfactant lipids. Compared to usually about 7% in healthy controls (Table 1), it is likely that the increased cholesterol concentration was mainly responsible for the biophysically defective surfactant. In addition, a relative reduction of surface active phosphatidylcholine (Table 1) and of the anionic phospholipids phosphatidylglycerol and phosphatidylinositol may have contributed to disease pathogenesis (38).

Our data clearly demonstrate aberration and inhibition of the pulmonary surfactant system in NPC2 disease. This information is of value not only for future diagnostic reference, but also for the development of therapeutic concepts for early lung disease in NPC2 patients. This means that pediatricians should anticipate the development of pulmonary alveolar proteinosis and prepare for therapeutic lavages very early on. We as well as the other two groups, who reported infants with NPC2-associated alveolar proteinosis, suggest that until now this treatment option was used too late in the course of the disease in order to be sufficiently effective, especially in view of the technically very demanding conditions for whole lung lavages in very young infants (17, 18, 39). This procedure has been successfully used in some older patients with other lysosomal storage diseases (35, 39, 40). Alternate strategies include the stimulation of alveolar macrophage phagocytosis of surfactant and are investigated in autoimmune pulmonary alveolar proteinosis (20, 41). In our infant, subcutaneous GM-CSF in pharmacological doses given for more than two months had no effect on the severity of the pulmonary alveolar proteinosis, whereas a profound effect on bone marrow, i.e. induction of eosinophilia was noted, indicating normal function of the GM-CSF transduction pathway.

In conclusion, the differential diagnosis of the respiratory symptoms of bronchiolitis or pneumonia in infants not responding to common treatments should include pulmonary alveolar proteinosis due to NPC2 disease. The features of this condition are recapitulated in the NPC2-hypomorph mouse model, where the typical

histology also developed long before the animals died. It therefore represents a highly attractive animal model to study novel therapeutic interventions for infants with NPC2 disease.

Conflicts of interest

The authors state that they do not have competing interests.

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