

Increased Risk of Interstitial Lung Disease in Children with a Single R288K Variant of ABCA3

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The *ABCA3* gene encodes a lipid transporter in type II pneumocytes critical for survival and normal respiratory function. The frequent *ABCA3* variant R288K increases the risk for neonatal respiratory distress syndrome among term and late preterm neonates, but its role in children's interstitial lung disease has not been studied in detail. In a retrospective cohort study of 228 children with interstitial lung disease related to the alveolar surfactant system, the frequency of R288K was assessed and the phenotype of patients carrying a single R288K variant further characterized by clinical course, lung histology, computed tomography and bronchoalveolar lavage phosphatidylcholine PC 32:0. Cell lines stably transfected with *ABCA3*-R288K were analyzed for intracellular transcription, processing and targeting of the protein. *ABCA3* function was assessed by detoxification assay of doxorubicin, and the induction and volume of lamellar bodies. We found nine children with interstitial lung disease carrying a heterozygous R288K variant, a frequency significantly higher than in the general Caucasian population. All identified patients had neonatal respiratory insufficiency, recovered and developed chronic interstitial lung disease with intermittent exacerbations during early childhood. *In vitro* analysis showed normal transcription, processing, and targeting of *ABCA3*-R288K, but impaired detoxification function and smaller lamellar bodies. We propose that the R288K variant can underlie interstitial lung disease in childhood due to reduced function of *ABCA3*, demonstrated by decelerated detoxification of doxorubicin, reduced PC 32:0 content and decreased lamellar body volume.

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INTRODUCTION

Interstitial lung diseases (ILD) encompass a large number of diffuse parenchymal lung disorders involving the lung parenchyma and the alveolar

interstitium (1). The disease spectrum is much broader in childhood, as many diseases primarily manifest during infancy (2). The surfactant dysfunction disorders are the best molecularly

characterized subgroups of ILD (3–5), and among these, variants in the ATP-binding cassette sub-family A member 3 (*ABCA3*) are the most frequent cause (6–8).

The carrier frequency of heterozygous *ABCA3* variants in the population predicts disease prevalence of 1 in ~3,100 of European descent and a total of ~750 homozygous newborns annually in the United States (9). This incidence is in the range of that for cystic fibrosis (about 1 in 2,500 for European descent infants) (10). However, sick patients carrying *ABCA3* variants are not as frequently identified as could be expected based on these numbers. The main reason for this is the

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relatively large, and thus costly, analysis of the *ABCA3* gene, which is assessed mainly in very severe, frequently lethal courses of respiratory distress syndrome in the mature neonate (11,12). Additional reasons include many not, or only poorly, defined *ABCA3* variants with unclear pathogenic significance (9,13). Some variants lead to rare but well-characterized chronic children's interstitial lung disease with a firm histopathological pattern including desquamative interstitial pneumonitis or nonspecific interstitial pneumonitis (14,15). The *ABCA3* variant R288K (c.863G>A) has been previously associated with pediatric respiratory disease (6) and a single copy was three- to four-fold enriched among the European-descent infants with neonatal respiratory distress (9,16,17), although it is predicted to be benign and tolerated by the algorithms PolyPhen and SIFT.

ABCA3 is a lipid transporter in alveolar type II cells, where it localizes to the outer membrane of lamellar bodies (LBs) which store surfactant. Variants in the *ABCA3* gene can impair the intracellular localization, processing and transport activity of the *ABCA3* protein. For instance, *ABCA3* with the variants L101P, L982P, L1553P, Q1591P and Ins1518fs/ter1519 was retained in the endoplasmic reticulum, whereas N568D, G1221S and L1580P mutant *ABCA3* proteins were correctly localized in the LB limiting membrane, but showed decreased ATP hydrolysis activity compared with wild-type protein (12). *ABCA3* activity is also linked to LB biogenesis. Heterozygous *ABCA3*^{+/-} knockout mice had fewer LB in their lungs (18). Radiolabeled substrates, which were incorporated into newly synthesized disaturated phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine molecules, were lower in both LB and surfactant when compared with *ABCA3*^{+/+} mice (18). Additionally, inherited *ABCA3* variants caused a decrease of total phosphatidylcholine

content in bronchoalveolar lavage of infants and raised surface tension compared with control group (19,20). Molecular species patterns of phosphatidylcholine, that is, dipalmitoylphosphatidylcholine (PC 32:0), were also decreased in lavage of affected children (20).

In this study we investigated the hypothesis that the *ABCA3* variant R288K may be permissive for children's interstitial lung disease. Supportive of such a connection would be an increased frequency of this variant in a population of patients affected by ILD over the general population, *ex vivo* evidence from these subjects for a certain functional impairment related to *ABCA3* and *in vitro* data from cells expressing this variant and proving functionally concordant cellular abnormalities.

MATERIALS AND METHODS

Cohort

Between 2003 and 2012, 377 infants and children were diagnosed in the program for rare lung diseases of the Kids-Lung Register as suffering from ILD related to the alveolar surfactant region on a clinical, radiological and sometimes histological basis (21). Of these patients, 228 were sequenced for variants in the *ABCA3* gene. In 62 patients possible disease causing variants were identified and R288K was present in 11 cases. The latter patients were analyzed in more detail (Supplementary Tables 1, 2, 3). Two of these children (patients 10 and 11 in Supplementary Table 1) had additional damaging homozygous *ABCA3* variants very likely to cause *ABCA3* insufficiency and determine their clinical course. These subjects were considered separately in further analysis as we intended to understand the role of heterozygous R288K alone or in association with nondamaging variants. As control cohorts for the sequence variant frequencies the Caucasian collections were used (National Heart, Lung and Blood Institute Exome Variant Server [EVS]).

Ethics Statement

All parents or guardians of the children gave their informed consent; an older youth assented. The retrospective analysis of the data was approved by the institutional review board (EK 026-06) of the University of Munich. Prospective collection and analysis of data was performed under (EK 257-10) and the FP7-305653 project chILD-EU (EK 111-13).

Genetic Analysis for *ABCA3*, *SFTPC*, and *SFTPB*

Patients were investigated for variants in exons 4 to 33 of the *ABCA3* gene (NM_001089), in exons 1 to 11 of the *SFTPB* gene (NM_000542) and in exons 1 to 5 of the *SFTPC* gene (NM_003018) (22). Genomic DNA were amplified by PCR using the primers listed (Supplementary Table 4). Electrophoresis of sequenced products was performed on an Applied Biosystems 3130xl Genetic Analyzer.

Plasmids

pT2/HB transposon vector was purchased from Addgene (plasmid #26557). First, the puromycin resistance gene with corresponding PGK promoter elements was amplified from pLKO.1puro vector (purchased from Addgene, plasmid #8453) and cloned into the pT2/HB vector using *EcoRI* and *HindIII* restriction sites, yielding pT2/HB-puro. Second, *ABCA3* cDNA fused to a C-terminal HA-tag was amplified with corresponding cytomegalovirus (CMV) promoter elements from pcDNA4TO-*ABCA3*-HA vector and cloned into pT2/HB-puro using *NheI* and *NotI* restriction sites, yielding pT2/HB-puro-*ABCA3*-HA. The R288K point variant was introduced into pT2/HB-puro-*ABCA3*-HA using the Q5[®] site-directed mutagenesis kit with the following primers: R288K-for 5'-GAGAA GGAAA AGAGG CTGAA GGAGT AC-3' and R288K-rev 5'-CTGCA CGACA GCACG GGC-3'. pCMV(CAT)T7-SB100 transposase was ordered from Addgene (plasmid #34879). All resulting vector constructs were verified using Sanger sequencing. Sequence analysis and alignment was done using Clone Manager Suite (Version 6.00).

Antibodies

The following antibodies were used: rabbit anti-HA, rat anti-HA, mouse anti-CD63, mouse anti-calnexin, chicken horse radish peroxidase (HRP)-conjugated anti- β -Actin, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG.

Cell Culture

Human A549 and HEK293 cells were purchased from the German Collection of Microorganisms and Cell Cultures. Both cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂.

HA Generation of Single Clones Stably Expressing ABCA3-HA

To evaluate the effect of the R288K point variant *in vivo*, ABCA3-HA wildtype (WT) and mutated protein were stably transfected using the *Sleeping Beauty* transposon system into A549 cells and HEK293 cells, respectively. Therefore cells were seeded into six-well plates and cotransfected with pCMV(CAT)T7-SB100 and pT2/HB-puro-ABCA3-HA WT or R288K respectively in the ratio of 2:5 using X-tremeGENE HP DNA transfection reagent. Forty-eight hours posttransfection, selection of stable cells was started by addition of 1 μ g/mL puromycin. Single cell clones were obtained by transferring single cells into the wells of a 96-well plate.

Immunofluorescence Staining

Stable A549 cells grown in eight-well slides were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. After treating with 0.1 mol/L glycine in PBS, cells were permeabilized with 0.1% Triton X-100 for 10 min. Unspecific binding sites were blocked with 3% bovine serum albumin and 10% FCS in PBS for 30 min. Labeling with primary antibodies was carried out for 1 h at room temperature. Cells were washed three times with PBS and stained with the appropriate Alexa Fluor secondary antibodies. After washing three times with PBS, cells were stained with 0.1 μ g/mL DAPI for 10 min and washed with PBS. The cells were immediately covered with mounting medium

(90% glycerine in phosphate-buffered saline and 2% DABCO). Objects were viewed with an Olympus IX81/Fluoview FV1000 confocal laser scanning microscope and imaged with the Olympus Fluoview version 4.2 software.

Real-time PCR

Total RNA was collected from cells grown for 48 h in six-well plates using the High-Pure RNA Isolation Kit according to the manufacturer's instructions. RNA concentrations were determined with a NanoDrop spectrophotometer. One microgram of total RNA was transcribed into cDNA with the Tetro cDNA Synthesis Kit. Real-time PCR was carried out on a 7900HT Fast Real-Time PCR System using SYBR green and the following primers: ABCA3-for 5'-CATGG TCAGC ACCTT CTTCA-3', ABACA3-rev 5'-TTCTG GCTCA GAGTC ATCCA-3', HPRT-for 5'-GCTGA CCTGC TGGAT TAC-3' HPRT-rev 5'-TGCGA CCTTG ACCAT CTT. Relative changes in gene expression caused by ABCA3-WT or R288K variant were calculated using the 2^{- $\Delta\Delta$ CT} method with hypoxanthine-guanine phosphoribosyltransferase (HPRT) as housekeeper.

Immunoblotting

Cells were seeded in six-well plates for 48 h and then rinsed with PBS once and subsequently trypsinized and again washed with PBS. After that, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing 0.15 mol/L sodium chloride, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 5 mmol/L ethylene diamine tetraacetic acid (EDTA) and 50 mmol/L Tris pH 8. RIPA buffer was accomplished by complete protease inhibitor. The lysate was centrifuged for 30 min at 1,000 \times g and 4°C. The protein concentration of the post-nuclear supernatant (that is, whole-cell lysate) was determined with the Pierce BCA protein assay using bovine serum albumin as the protein standard. In total, 20 μ g of cell lysates in 4 \times LDS buffer were loaded onto NuPage Mini Tris-Acetate gels.

Following gel electrophoresis, proteins were blotted to polyvinylidene fluoride membranes. After transfer, membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween (TBS-T). Membranes were incubated overnight with primary antibodies in blocking solution. After washing the membranes three times with TBS-T, HRP-conjugated secondary antibodies were applied 1 h at room temperature. Detection was performed using ECL reagent.

Viability Assay

Cell viability was assessed by determining cleavage of 2,3-bis (2-methoxy-4-nitro-sulphophenyl)-2H-tetrazolium-5-carboxyanilide in the presence of phenazine methosulfate. For XTT assay, cells were seeded in 96-well plates in RPMI medium containing 10% FCS and no phenol red. After different points of time, absorbance was measured at 490 nm and 650 nm using a spectrophotometer.

Cytotoxicity Assay

Triplicates of A549 cells stably transfected with ABCA3-WT and ABCA3-R288K and cells transfected with mock control were seeded into six-well plates. After an incubation time of 96 h, cell supernatants were harvested and the amount of the lactate dehydrogenase (LDH) enzyme was quantified colorimetrically at 490 nm using the CytoTox 96[®] Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions. The resulting optical densities were referred to numbers of cells counted with the Cellometer Auto T4 Cell Viability Counter.

Lipid Analyses of Human Bronchoalveolar Lavages

After protein level determination, lipids of bronchoalveolar lavages (BALs) obtained from the patients were analyzed by ESI-MS/MS in positive ion mode as described in (20).

Doxorubicin Assay

For doxorubicin assay HEK293 cells stably expressing ABCA3 wild-type

protein or R288K variant were seeded as octuplicate in 96-well plates at a density of 1×10^5 cells per well. After attaching to the 96-well plate HEK293 cells were treated with different concentrations of doxorubicin for 3 h. Cells were washed subsequently with PBS and incubated for further 24 h in RPMI medium containing 10 % FCS and no phenol red under normal conditions. Viability was measured with XTT assay (as previously described).

Electron Microscopy

Cells were fixed in 150 mmol/L HEPES, pH 7.35, containing 1.5 % formaldehyde and 1.5 % glutaraldehyde at room temperature for 30 min and then at 4°C overnight. After dehydration in acetone, cells were embedded in EPON. 50 nm sections were stained with 4 % uranyl acetate and lead citrate and observed in a Morgagni TEM. Images were taken with a 2 K side mounted Veleta CCD camera.

Calculation of Vesicle Volume

A549 cells containing either ABCA3 wild type or R288K protein variant were seeded in eight-well slides. After an incubation time of 48 h, cells were fixed as described above and immunostained against HA-tag. Cells were imaged with the confocal laser scanning microscope. Diameter of each ABCA3-HA containing vesicle was determined with ImageJ (NIH; <http://rsb.info.nih.gov/ij/>) software tool. Vesicle volume was calculated with formula: $V = \frac{4}{3} * \pi * r^3$. Sixty different ABCA3-HA containing vesicles were randomly chosen from three distinct experiments, thus analyzing 20 vesicles per condition and experiment.

Statistical Analyses

Dependency on gestational age of the different ABCA3 variants was analyzed using Fisher exact test. Comparisons of two groups were done using Student *t* test. Comparisons of multiple groups were done using one-way repeated measure ANOVAs with Tukey *post hoc* test. Doxorubicin assay, phospholipid (PL)

classes and phosphatidylcholine (PC) species were analyzed using two-way repeated measure ANOVAs with Sidak *post hoc* test. Results were presented as mean \pm SEM of a minimum of three different experiments. *P* values <0.05 were considered to be statistically significant. All tests were performed using Graph-Pad Prism 6.0.

All supplementary materials are available online at www.molmed.org.

RESULTS

Clinical Characterization of Children with Interstitial Lung Disease and R288K

In our cohort of 228 children with ILD and ABCA3 sequenced, we identified 11 subjects as carriers of R288K. This frequency of R288K was clearly enriched over that in the general Caucasian population (71 R288K in 4,229 Caucasian subjects of the Exom variant server (National Heart, Lung and Blood Institute Exome Variant Server) (*P* = 0.0014)). We next analyzed the dependency on gestational age of the different ABCA3 variants identified. Whereas all the other possibly disease causing ABCA3 variants were evenly distributed among mature and immature infants, R288K was found more frequently in premature infants (Table 1).

All our patients carrying a single heterozygous R288K variant were Caucasians, had nonconsanguineous parents and did not carry known disease-causing genetic variants in *SFTPC* and *SFTPB* (Supplementary Table 1). In all

children, the initial manifestation of lung disease was during the neonatal period, only five were premature infants (median gestational age 30 wks, range 27 to 32). Six children were mechanically ventilated; most recovered and had chronic or intermittent episodes with respiratory symptoms during infancy and childhood. Those old enough to perform lung function tests had restrictive lung disease (Supplementary Table 2). The computed tomography pattern was consistent with ILD in six of the nine children. In two where no CT scan was available, histology documented ILD. Six of the nine children had lung biopsies, always showing ILD alone or in combination with emphysematous areas (Supplementary Table 3).

No Evidence for Impaired ABCA3 Transport Activity in Children with Chronic ILD and Heterozygous for R288K

Initially, we excluded severe ABCA3 deficiency as the molecular cause of the diseases by excluding complete ABCA3 deficiency and demonstrating evidence for some ABCA3 function (Table 2). The subjects had regular ABCA3 histopathologic staining (available in four subjects), had normally formed lamellar bodies (available in three subjects) or had a dipalmitoyl-phosphatidylcholine (PC 32:0) content in bronchoalveolar lavage (in six subjects available) that was on average a little higher than typically found in infants with ABCA3 deficiency, but significant lower than in

Table 1. Frequencies of ABCA3 variants among 228 neonates and infants (mean gestational age 35.4 wks) sequenced for ABCA3 variants and interstitial lung disease.

Gestational age (wks)	<37		≥37		
Mean gestational age (wks)	31.6	31.3	39.4	39.4	
ABCA3 variant present	Yes	No	Yes	No	P ^a
Any variants	15	48	47	118	0.5106
R288K	6	8	5	43	0.0012
R288K without other disease causing ABCA3 variant (n = 2)	6	8	3	43	0.0001

^aFisher exact test

Table 2. *Ex vivo* evidence for functionality of ABCA3 transport activity.

Patient	Code ^a	Immunohistologic staining for ABCA3	Electron microscopy	Dipalmitoyl-phosphatidylcholine (PC 32:0) (% total PC) in BAL	Overall evidence for ABCA3 transport activity
Expected normal result		Type 2 cells with granular ring-like structures	Concentric lamellar	45.8 ± 3.6 ^b	
1	199	NA	Yes, normal	40.8	Yes
2	652	NA	NA	40.7	Yes
3	1300	Yes, normal	Yes, normal	41.1	Yes
4	1468	Yes, normal	NA	42.1 ^c	Yes
5	1479	Yes, normal	NA	23.5	Yes, marginal
6	1601	NA	NA	NA	Yes, no investigations as mild course
7	2253	Yes, normal	Yes, normal	30.6 ^c	Yes
8	2405	NA	NA	NA	Yes, no investigations as mild course
9	2741	NA	NA	NA	Yes, no investigations as mild course

NA, not available

^aGriese, Kiermeier *et al.* 2015.

^bDipalmitoyl-phosphatidylcholine (PC 32:0) was 12.7 ± 4.2 % of total PC in four subjects with severe neonatal disease causing ABCA3 variants.

^cLung tissue.

healthy children (Figure 1). In three other subjects, no such investigations were done due to mild and improving course.

It was of interest that all patients heterozygous for R288K presented to us as infants or children, rather than as neonates, with an unusual course of ILD, and which their treating physicians could not explain by prematurity or perinatal lung injury. All surviving children improved, but their ILD was not cured at follow up. Despite broad clinical investigations, no other causes of their ILD could be identified.

To summarize, these children with chronic ILD had enrichment of the R288K variant in ABCA3 and somewhat reduced dipalmitoyl-phosphatidylcholine in the alveolar space, that is, strong epidemiologic and biochemical evidence for an involvement of ABCA3 in their pathogenesis. Although the R288K variant was predicted not to be detrimental to protein function by SIFT and Polyphen, our data nonetheless suggest impairment of ABCA3 function due to the presence of this particular variant. Therefore, we performed detailed studies on the cellular effects of the R288K variant.

Transcription, Intracellular Processing, and Cellular Targeting of R288K in ABCA3-transfected Lung Cells

Stable transfection of A549 cells with DNA coding for ABCA3-WT or ABCA3-R288K induced ABCA3-mRNA

transcription of 200-fold compared with mock transfected cells (Supplementary Figure 1A). Intracellular processing as assessed by Western immunoblotting did not differ between ABCA3-WT and ABCA3-R288K. Both proteins were present

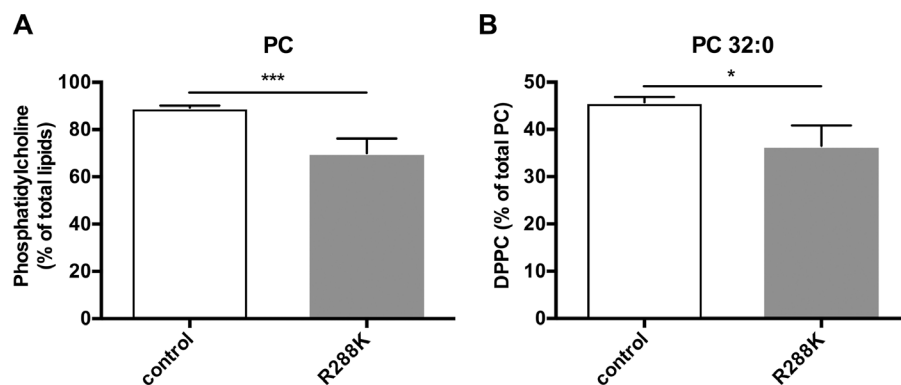


Figure 1. Levels of phosphatidylcholine and dipalmitoyl-phosphatidylcholine in bronchoalveolar lavage fluid of infants heterozygous for R288K in ABCA3. (A) Phosphatidylcholine of patients carrying heterozygote R288K variant (n = 4) compared with healthy control group (n = 11). Data are shown in % of all analyzed PL classes measured in BAL. (B) Dipalmitoyl-phosphatidylcholine amounts of healthy patients (n = 11) and patients with R288K variant (n = 4) measured in BAL. Data are shown in % of total PC. All results were expressed as mean ± SEM. **P* < 0.05; ****P* < 0.001.

as the 190 and 170 kDa processing forms (Supplementary Figure 1B) and their intensity ratios did not differ (Supplementary Figure 1C). Cell growth and proliferation were unaffected in comparison to mock controls (Supplementary Figure 1D, E). Intracellular localization was investigated by immunofluorescence staining and revealed normal colocalization of both the wild type and the R288K mutated ABCA3 protein with CD63, a marker for late endosomes and lamellar bodies (Supplementary Figure 1F). Obvious protein misfolding was ruled out by lack of colocalization with the ER-resident chaperone calnexin (Supplementary Figure 1G). These findings were confirmed independently in HEK293 cells. Whereas there was no difference in protein expression, mRNA expression differed between ABCA3-WT and ABCA3-R288K expressing cells (Supplementary Figure 2).

Impaired ABCA3-Dependent Doxorubicin Detoxification in R288K Variants

ABCA3 transport activity was analyzed in HEK293 cells stably transfected with ABCA3-WT or ABCA3-R288K by means of their ability to detoxify doxorubicin (Figure 2). High activity of the transporter, that is, overexpression of ABCA3 protein in childhood acute myeloid leukemia patients, induced resistance against doxorubicin (23). HEK293 cells stably expressing R288K mutated ABCA3 protein had a diminished viability compared with cells expressing WT protein when treated with 5, 10 or 20 μmol/L of doxorubicin respectively (Figure 2A). In concordance, the morphology of the cells expressing ABCA3-R288K was altered to a round-shaped appearance compared with ABCA3-WT expressing cells (Figure 2B; 10 μmol/L doxorubicin). These results point to a reduced ABCA3 transport activity as a result of the R288K variant.

Lamellar Bodies of ABCA3 with the R288K Variant

Electron microscopy of A549 cells stably expressing ABCA3-WT

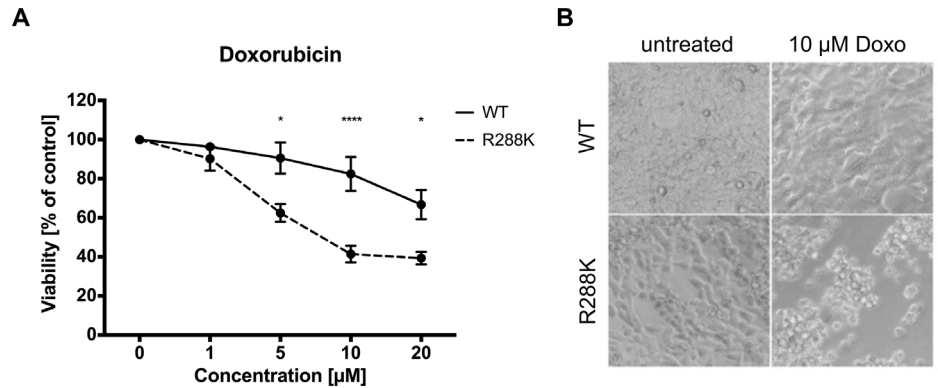


Figure 2. Induction of cellular multidrug resistance *in vitro* by ABCA3. (A) Effects of soluble doxorubicin on viability of HEK293 cells stably expressing ABCA3-WT or ABCA3-R288K protein. Different concentrations of doxorubicin were used and viability was measured with XTT assay. Values are displayed as means ± SEM of HEK293 cells stably expressing either ABCA3 wild-type protein (solid line) or R288K mutated ABCA3 protein (dashed line). **P* < 0.05; *****P* < 0.0001. (B) Light microscopy of HEK293 cells stably expressing ABCA3-WT and ABCA3-R288K protein respectively. Additionally cells were treated with 10 μmol/L doxorubicin for 3 h and displayed after 24 h of growth in RPMI medium supplemented with 10% FCS under normal conditions.

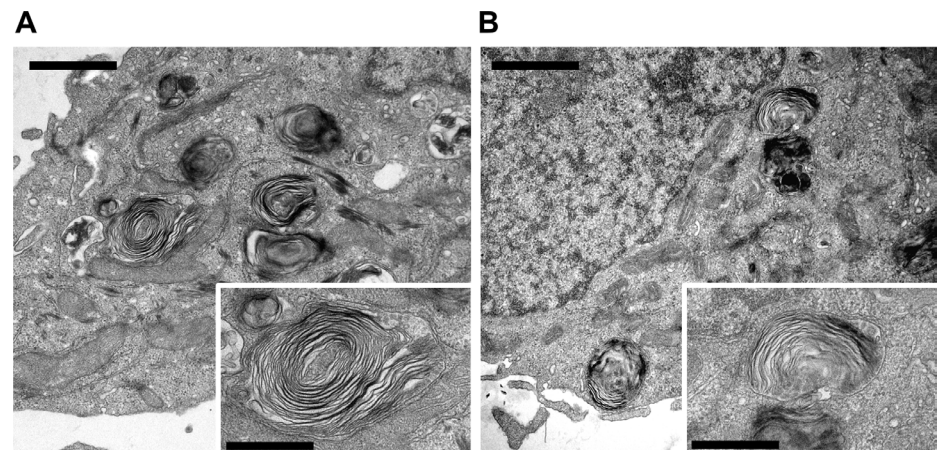


Figure 3. Transmission electron microscopy. Electron microscopy of A549 cells expressing ABCA3-WT (A) or ABCA3-R288K (B). Several lamellar bodies are visible in the overviews. These exhibit concentric lamellae, as distinguishable in the higher magnifications (insets). Scale bars are 1 μm for overviews and 0.5 μm for insets.

or R288K mutated protein revealed an induction of lamellar body (LB)-like structures in A549 cells (Figures 3A, B). LB-like structures of both cell lines showed well-formed organelles containing concentric membranes arranged in parallel. Volumes of LB-like structures in cells expressing ABCA3-R288K were quantitatively compared with cells expressing ABCA3-WT protein. Using immunofluorescence staining of A549

cells stably expressing ABCA3-R288K, smaller ABCA3-positive vesicles were found compared with cells expressing ABCA3-WT protein (Figure 4).

DISCUSSION

In this study we present a cohort of children with chronic ILD characterized by their carriage of a single R288K variant of ABCA3. All children suffered from a mainly restrictive lung disease

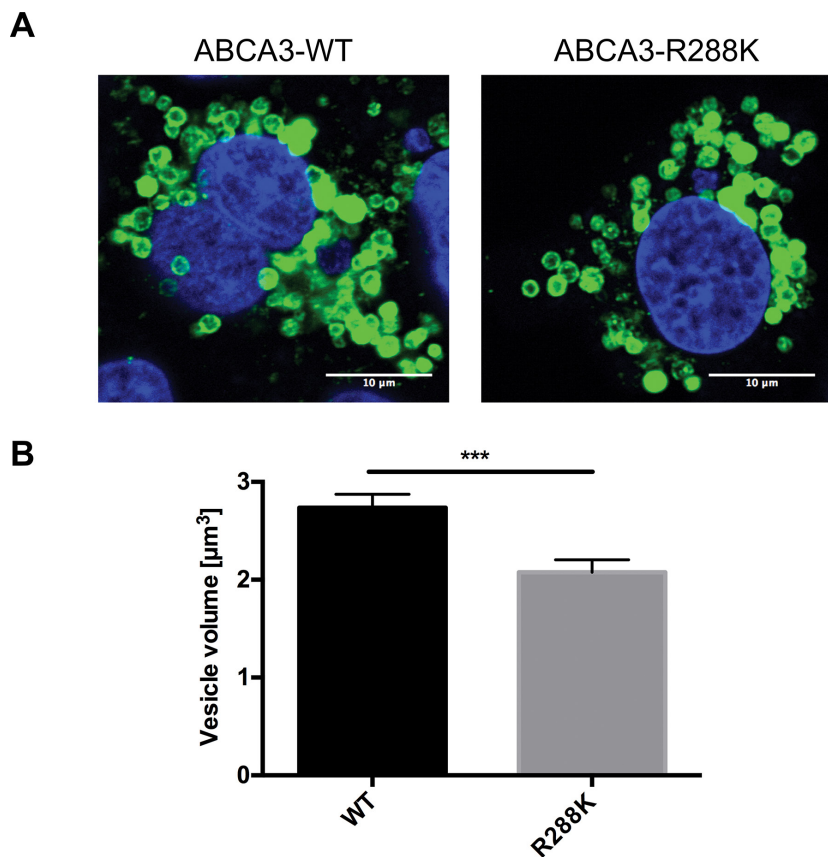


Figure 4. Determination of vesicle volumes in A549 cells with ABCA3-WT or ABCA3-R288K protein. (A) Immunofluorescence staining of HA-tagged ABCA3 vesicles imaged with a confocal microscope. Shown is either ABCA3-WT (left) or ABCA3-R288K containing vesicles (right). (B) Volumes of ABCA3 containing vesicles harboring either ABCA3-WT or ABCA3-R288K were calculated on the basis of vesicle diameters determined with ImageJ software tool. *** $P < 0.001$.

with the typical histological and chest CT imaging pattern of chronic pediatric ILD. However, they did not exhibit the generic features of complete ABCA3 deficiency. We present evidence that the presence of the R288K variant interferes with ABCA3's transport activity. Therefore, we propose that the development and long-term persistence of chronic ILD in our cohort was due to the presence of a single R288K variant of ABCA3 and not a result of their neonatal history of respiratory distress following either term or preterm birth.

Manifestation of ABCA3 deficiency due to homozygous or compound heterozygous variants causes severe respiratory distress in mature infants,

frequently leading to early death (11,12) or chronic ILD in a small number of older children and young adults (14,15). In this study, we characterize subjects carrying the R288K variant on a single allele and suffering from a characteristic chronic pediatric ILD phenotype. Whereas the individual course of their lung disease was quite variable, all patients had a characteristic diffuse parenchymal lung disease. Histology of lung biopsies demonstrated ILD present with almost the entire spectrum of histologic patterns known for these conditions, including pulmonary alveolar proteinosis (PAP), nonspecific interstitial pneumonitis (NSIP), desquamative interstitial pneumonitis (DIP), chronic pneumonitis of

infancy (CPI), interstitial emphysema, dystelektasis and eosinophilia, often in combination. Such heterogeneity is not unusual, even in the presence of the same disease causing variants (2,4,21), and likely represents the morphologic response of lung parenchyma to variable injuries in different individuals.

Our data suggest that heterozygous R288K variants in ABCA3 are not strong enough determinants permitting the manifestation of ILD in the absence of additional factors. This is supported by the fact that only two out of nine parents carrying the variant had some, but not severe, respiratory symptoms. However, the precise nature of their conditions was not investigated to great detail. In our patients, neonatal respiratory distress was a consistent feature, together with injury from recurrent airway infections. The strong interaction of single ABCA3 variants and prematurity was previously demonstrated (17) and confirmed here. Other modifying factors may be present like cis-acting elements, introns or nontranslated regions within the ABCA3 gene, which were not covered by our sequencing strategy (24). Whereas variants in *SFTPC* or *SFTPB* were excluded, variants in other genes not directly related to ABCA3 expression, epigenetic regulation and additional environmental or developmental factors are also conceivable. Furthermore, previous studies showed that some ABCA3 variants had a mild cellular effect, associated with increased inflammation (25).

Lower levels of phosphatidylcholine and dipalmitoyl-phosphatidylcholine in the alveolar space of the infants are likely to be a result of functional impairment of ABCA3-R288K. The other phospholipid species (lyso-phosphatidylcholine, phosphatidylglycerol, sphingomyelin, phosphatidylethanolamine, phosphatidylserine) were not different. Additionally, a reduced capacity for detoxification of doxorubicin and a smaller volume of ABCA3-positive vesicles provide *in vitro* evidence for reduced ABCA3 transport function due to the presence of the R288K variant. Taken

together, these data point to a causative role of the R288K variant. Although the functional impairment of ABCA3-R288K is likely too small to induce lung disease on its own, when acting in combination with significant respiratory stress at birth or later in life during respiratory tract infections, its effects will become amplified and will cause notable clinical symptoms.

As there is a strong developmental dependency of ABCA3 expression with an increase toward birth (26), an increased vulnerability in premature infants is expected. The strong association of the R288K variant with respiratory distress obviously results from a partially defective surfactant lipid transport, which will have a more pronounced effect the more immature the baby.

Mice carrying *ABCA3* knockout on a single allele show an increased susceptibility to injury (27). Similarly, single point variants in the *ABCA3* gene can reduce the expression of mature protein, presumably by increasing endoplasmic reticulum (ER)-associated degradation of misfolded protein (28). Beyond the neonatal period, infants carrying a single R288K variant of ABCA3 may be more vulnerable to lung injury affecting the alveolar space, resulting in an increased risk of chronic pediatric ILD involving surfactant dysfunction. Among the most likely mechanisms triggering ILD in our patients were the frequently observed viral and other respiratory tract infections, which put an enormous burden on the ABCA3-dependent lung surfactant system. As demonstrated previously, and in agreement with our *ex vivo* measurements, infection with respiratory syncytial virus strongly impaired the expression of alveolar type 2 cell differentiation marker SP-C and other key epithelial cell proteins in the presence of ABCA3 variants. Simultaneously, a mesenchymal phenotype was acquired, indicating development of pulmonary fibrosis (29).

Our observations imply that the presence of a single mutated allele is permissive to cause symptoms in conjunction with common and usually well-tolerated burdens of the respiratory system. This is in concordance with findings in individuals

with single allele variants in another ABC transporter, that is, ABCA7 also called cystic fibrosis transmembrane regulator (CFTR). The expression of single alleles increases the risk for bronchiectasis (30,31) and rhinosinusitis (32), as well as extra pulmonary manifestations of cystic fibrosis like pancreatitis (33,34) or azoospermia (35). In the case of ABCA3-R288K, it remains to be elucidated whether the effects of the variants can be explained by haploinsufficiency alone or if the variant exerts a dominant-negative effect.

CONCLUSION

Whereas a major strength of our study is the detailed cellular analysis of the R288K variant, identifying mechanisms by which it may exert its disadvantageous effects, there are some weaknesses in our study including its retrospective design resulting in variable follow up, a relatively low number of cases due to the rarity of pediatric ILD and the fact that biopsies were obtained in many but not all cases. In addition, the cellular model is not perfect as clonal differences might account in part for the results; the transfection is homozygous since either ABCA3-WT or R288K variant were introduced and, although we used two different cell lines yielding similar results, these are still different from primary type II cells. On the other hand, this is a third and independent cohort demonstrating some pathogenic potential of R288K, showing for the first time a role in pediatric ILD, and providing detailed cell biological analysis.

Taken together, we show that the presence of the ABCA3-R288K variant on a single allele, when acting together with aggravating external stressors like prematurity or recurrent respiratory infections, can cause respiratory insufficiency and ILD. We also demonstrate that in spite of R288K having been predicted to be neutral by *in silico* prediction algorithms, this particular variant indeed may cause functional impairment of ABCA3 leading to clinically relevant consequences. This finding also highlights the notion that results from prediction algorithms for variants identified during analysis of next

generation sequencing data need to be backed by clinical and experimental data.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Supplemental Data

Increased Risk of Interstitial Lung Disease in Children with a Single R288K Variant of ABCA3

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Supplementary Table S1. Genetic data of the patients.

Patient	Sex	Ethnicity	Family history	ABCA3 sequence variant	SFTP B	SFTP C
1	Female	Caucasian	Mother 33 years, I grav, I para	R288K heterozygous	nl	nl
2	Female	Caucasian	Mother IV grav, III para	R288K heterozygous	nl	nl
3	Male	Caucasian (f) Pacific Islander Polynesian (m),	Mother, brother: bronchial asthma, Father: lung problems	R288K heterozygous	nl	nl
4	Male	Caucasian	Geminus I, 22 gest wk death of sibling	R288K heterozygous	nl	nl
5	Female	Caucasian	Parental smoking	R288K heterozygous	+/- Pos -384	nl
6	Male	Caucasian	Mother healthy, no lung symptoms	R288K heterozygous	nl	CC to GT sub-stitution at -92 to -91 to ATG start codon, heterozygous
7	Male	Caucasian	Twin died short after birth due to lung failure	R288K heterozygous, P766S heterozygous, (trans)	nl	nl
8	Female	Caucasian	Mother: hemithyroidectomy; father: bronchial asthma, suspected ILD, no biopsy taken	R288K heterozygous, S693L heterozygous (both cis)	nl	nl
9	Female	Caucasian	None	R288K heterozygous	n.d.	nl
10	Female	Caucasian	None	R43L/R288K+c4751 delT (frameshift) compound heterozygous (trans)	nl	nl
11	Male	African	None	Q215K homozygous, R288K homozygous	nl	nl

nl = normal, n.d. = not done

ABCA3 IN CHILDREN'S INTERSTITIAL LUNG DISEASE

Supplementary Table S2. Neonatal history, lung function and CT scan of the children with chronic interstitial lung disease and R288K in ABCA3.

Patient	Gest. age	Oxygen neo. period	Mech. ventilation neo. period	Onset lung disease (year)	Course lung disease	Lung function	CT scan
1	39	Yes	Yes	0.1	6 wks: tachydyspnea, cyanosis, wheezing;	N.a.	4 months: bilateral interstitial diffuse infiltration
2	36	Yes	Yes	0.0	Postpartal tachypnea; 6 months RDS due to bronchopneumonia; 8 months RDS due to UTI; bronchial hyperreactivity	7 years: FEV1 54%, FVC 56%	13 months: ground glass attenuation; 6 years: bronchopneumonia, hilar lymphadenopathy
3	32	Yes	No	0.1	RDS, persistent need of oxygen	N.a.	Interstitial changes, thickened septa, air trapping, emphysema
4	27	Yes	Yes	0.0	RDS	N.a.	N.d.
5	30	Yes	Yes	0.0; 4.3	RDS 3d ventilated, 8 d CPAP, 4.25 years dyspnea, cyanosis, coughing attacks, tachypnea, wheezing; 8 years rec. respir. infections	8 years: no desaturation under physical performance	4.5 years: honey comb pattern bilateral, ground glass attenuation; 7 years: bronchiectasis
6	30	Yes	No	0.0	Congenital lobar emphysema and interstitial lung disease	11 years: FEV1 58%, FVC 52%	3 months: emphysema and mosaic pattern in under-developed lung. 7 years: basal thickened and rarefied interlobar septa; mosaic perfusion; emphysema
7	29	Yes	Yes	0.0	RDS 26d ventilated, BPD, pulmonary hypertension	N.a.	N.a.
8	37	Yes	Yes	0.0	0.2 year: oxygen need, deteriorations of saturation; 1.8 years: obstructive episodes	N.a.	1 month: diminished ventilation, ground-glass attenuation bilateral
9	37	No	No	0.0	Tachydypnea since birth, 2.0 years recurrent bronchopulmonary infections	1 st lung function: LCI 9.5, central obstruction, lung compliance decreased 8.7ml/kPa/kg 2 nd lung function, at 7mo LCI normal, compliance improved 10 ml/kPa/kg	1 st CT: Mosaic pattern, ventilation inhomogeneities, no tracheobronchial malacia, ectasy of LAD-coronary artery 2 nd CT, at 7 mo, normal lung parenchyma, no ventilation inhomogeneity, constant ectatic LM, LAD, RCA after Kawasaki's disease
10	42	Yes	Yes	0.0	Postpartal tachydyspnea, cyanosis	N.a.	N.a.
11	40	No	No	0.0	No information	N.a.	N.a.

BPD = Bronchopulmonary dysplasia; CPAP = continuous positive airway pressure; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; LCI = lung clearance index; RDS = respiratory distress syndrome. N.a. = not available

Supplementary Table S3. Lung biopsy and other diagnosis, treatment, outcome of children with chronic interstitial lung disease and R288K in ABCA3.

Patient	Lung biopsy	Final pulmonary and additional diagnosis	Treatment	Follow up if alive or age of death (year)	Long term outcome
1	y	ILD with PAP pattern, mild lymphocytic infiltration	Prednisolone	0.4	dead
2	n	ILD with ground glass attenuation and septal thickening Arnold-Chiari malformation Typ II; recurrent airway infections	Prednisolone, antibiotics, long term oxygen (until age 7, now)	7.0	sick-better
3	y	ILD, Wilson Mikity Syndrome	Diuretics, inhalation, oxygen	3.9	sick-better
4	y	ILD / BPD, interstitial emphysema, dystelectasis, bronchiolitis	Surfactant	0.1	dead
5	y	ILD with NSIP, DIP pattern; eosinophilia	Hydroxychloroquine, azathioprine until 6.5 years; steroid boosts until 8 years, azithromycin	8.0	sick-better
6	y	Thickened and rarefied interlobar septa; mosaic perfusion; emphysema; dysalbuminemic hyperthyroxinemia, short stature	Inhaled steroids, diuretics, systemic steroids, theophylline; somatotropin	9.0	sick-better
7	y	ILD with CPI, NSIP, and DIP pattern	Oxygen, corticosteroids	0.5	dead
8	n	ILD, chronic respiratory symptoms; has healthy twin	Surfactant, dexamethasone; 1.8 years: montelukast, salbutamol	3.0	sick-better
9	n	ILD with ground glass attenuation, Kawasaki syndrome, aneurysm LCX	Oxygen, aspirin	4.0	sick-better
10	y	ILD previously published (case 3)#	Oxygen, mechanical ventilation	0.2	dead
11	y	ILD previously published (case 5)#	Oxygen, mechanical ventilation	0.3	dead

BPD = Bronchopulmonary dysplasia; CPI = chronic pneumonitis of infancy; DIP = desquamative interstitial pneumonitis; NSIP = non-specific interstitial pneumonitis; ILD = interstitial lung disease; PAP = pulmonary alveolar proteinosis; RDS = respiratory distress syndrome.
Brasch et al 2007.

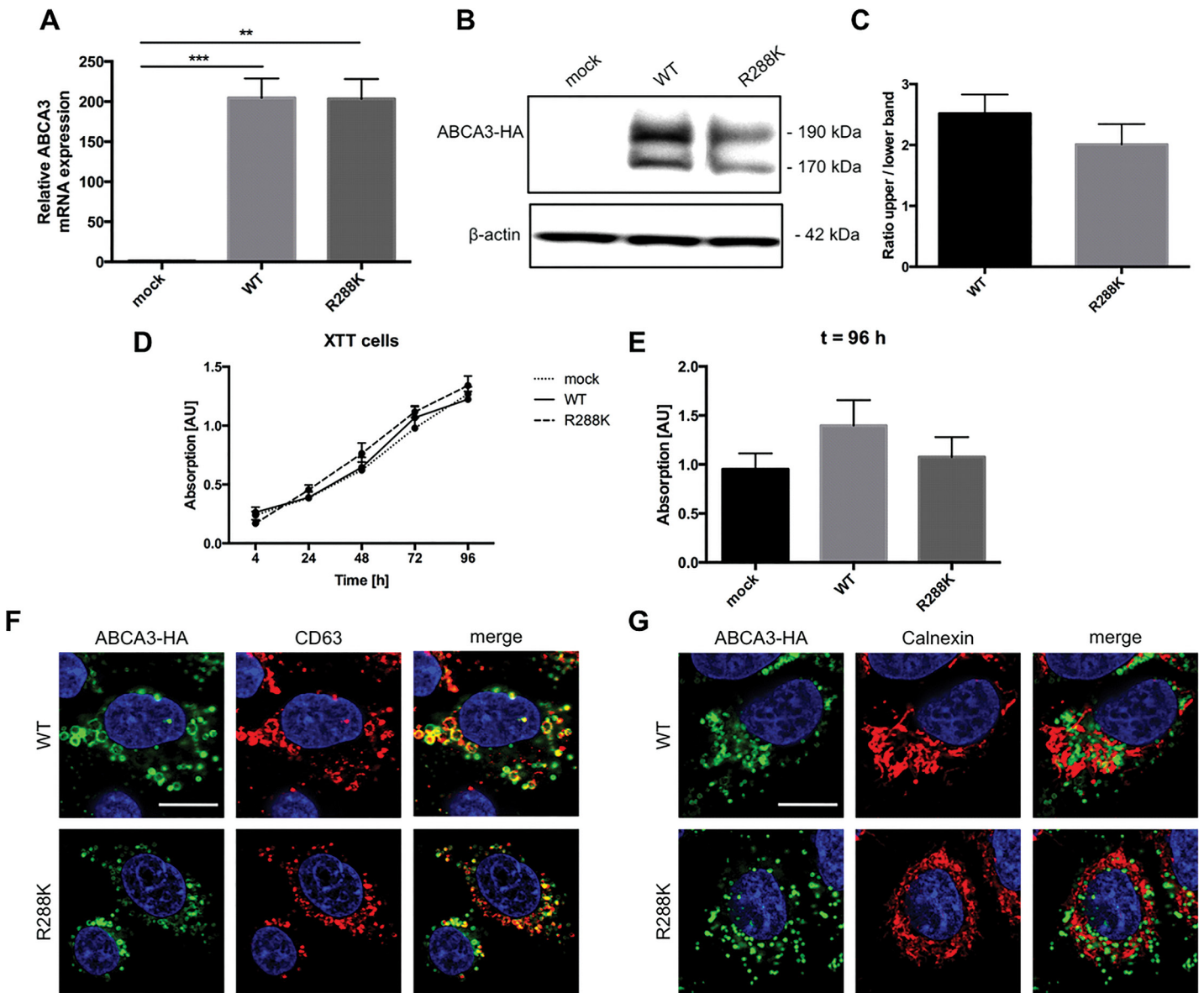
Supplementary Table S4. Primers used for analysis of ABCA3, SFTPC and SFTPB. The sequence of all PCR primers is given in the 5' to 3' direction.

ABCA3 gene	
A3-4-1	CCAAATCCCCACTCTGCGTG
A3-5-2	CAGCTGCTTCGCACATCCTG
A3-6-1	CAAAGCCCTAGAGGATTGCC
A3-6-2	CAGACCCAAAGGAGTGACTGC
A3-7-1	CTCTCCCACTCCACCCTGTTG
A3-7-2	CTGCTATAAGGACACATGCACACG
A3-8-1	TCTCATTGCTGTCAGTGTGTGG
A3-8-2	CTAAAACACCAAGCCTTGGACATG
A3-9-1	CTGCTGGGACAGTCGGACTC
A3-9-2	CTGACCATCCCTGGTCACAGG
A3-10-1	CTCTGGGAAGAAGTTTGTGGTCAG
A3-10-2	GCTGACTTTCCTCCTCCAGTCC
A3-11-1	GTGTAGATGGCAAGTGCCAGGAG
A3-11-2	CAGCTATCCAGCCCACACTCAG
A3-12-1	CATGCCAACCAAGCAGTGG
A3-12-2	CTCTCTCTGAACCAGTCCCAAGG
A3-13-1	CTGCATGGCTGTGTGCATCTAG
A3-13-2	CTATGAGGTCTCACTGCCGTGC
A3-14-1	CTAGGCTTGGTTCCTTCTGAGACG
A3-14-2	GTGCATCTCCTGCCGCTGTG
A3-15-1	CAGGGTCCTCAGAGGAAATTAGG
A3-15-2	CTCAGAACCCTGGCTCCTGC
A3-16-1	CAGCTACGTCAAGGAGAGGTTCC
A3-16-2	GCTCGTCCAGTATCAGCACCTG
A3-17-1	CCATCCTTGGAGGACTCAAGC
A3-17-2	CAGAGGCAACAGACAGGAAGTCTAG
A3-18-1	CAAGACACATTCTGCTTCAGC
A3-18-2	GCAGATTCATCTGGGCTGATG
A3-19-1	GTTCAAGTGTCTCCTGCCTCTG
A3-19-2	CTGGGCAACTAGAGTGAACTCC
A3-20-1	CATAAGCAGATGCATGAGCAAGC
A3-20-2	CTCGCAGACTCTCCTCTGCATG
A3-21-1	GCTGGCGTCACACAGAACAG
A3-21-2	CATTCGGAACAGCCAAGAACC
A3-22-1	GTCCCTGATTAGCCATGCTCAG
A3-22-2	GCAGACACAATGCTCTATCTATGGG
A3-23-1	GTGCTTGTGCTCTCCATAAGC
A3-23-2	CAGCTGGTCCGGTTCTGC
A3-24-1	GTCTGAGGACCTCCAAATGCTC
A3-24-2	CATGAACTGGGCCCATTC
A3-25-1	CTCCACACAGCACGGATAAGG
A3-25-2	CCACTCAGACGCAGAGGAGC
A3-26-1	GTCTGCCATGTCGCTCATGG
A3-26-2	GAGACCATCTGGTGCAGGAGC
A3-27-1	GATTGGGACGAGAAGCCTTG

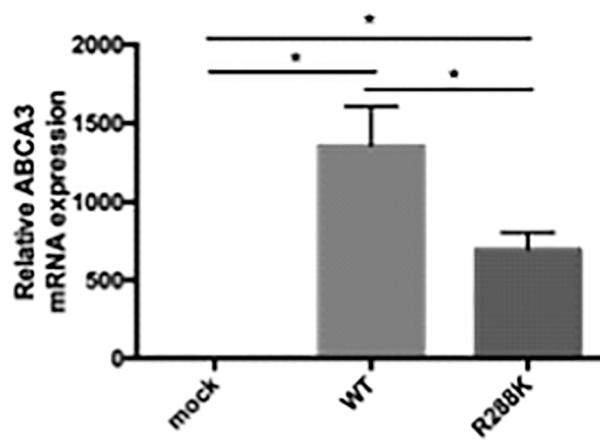
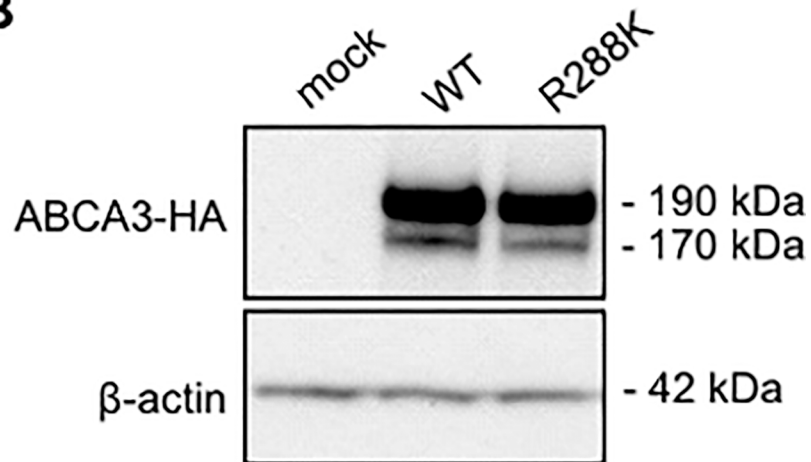
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Supplementary Table 4. *Continued.*

ABCA3 gene	
A3-27-2	GCAAAGCAGAGCAGTCTGAGC
A3-28-1	CTGATTATCAAGGAGCTCTCCAAGG
A3-28-2	CAAGCCACAGATGCAGCAGC
A3-29-1	CACTGGCAGGAACCACAG
A3-29-2	CTCCATCCTGGAGCCACAAG
A3-30-1	CTGTTCTGCAATTGCTGGGTG
A3-30-2	GACTCTGCACCAGATGCTGATG
A3-31-1	GAGAGCCAATGCCTTCCTGTC
A3-31-2	GTGCTCAGCACTGGAGTCCTC
A3-32-1	GAGGACTCCAGTGCTGAGCAC
A3-32-2	GTGACTCCTCTGTGGAAAGAGCC
A3-33-1	CTATTGCCAGAGGACTCCCAGG
A3-33-2	GAGTGCCTGGAGAAATCAACC
SFTP gene	
SB-1-1	GCCACAGAAATCTGTCTCTGACTC
SB-1-2	TTACCACCTGTCCTGGCTGATG
SB-2-1	GTTGGAGGAAGCCACAAGTCC
SB-2-2	GTTAATGCCTAGCACAAAGCAGTG
SB-3-1	AGAGCTGTCTGACGTCACATG
SB-3-2	ATCGCATCCATCCCATTCC
SB-4-1	AGCTGCATGTGCCTTGGAGTG
SB-4-2	CTCAGCTCTCCCTGCTCTGTC
SB-5-1	TGCCAACTACTTAACTCCTTGGC
SB-5-2	CTCCCCATGGGTGGGCACAG
SB-6-1	AGTGGTCCCTGAGCCCTTG
SB-6-2	GCTTCGGAGGTGGCTCTAGC
SB-7-1	CTAGAGCCACCTCCGAAGCC
SB-7-2	GTGTGAGTTGGGAGAGAGGTGG
SB-8-1	CTGGACTCTCTGATCCCCAGTG
SB-8-2	CAGAGGTGTTGGTTTCTGTCTC
SB-9-1	TCTGTGGTCCCCTGCAATG
SB-9-2	ACATCCTGTCTGCCTGTCTGTG
SB-10-1	TGATCCAGAGATGTGGAGAGGC
SB-10-2	GAGTGAGGCCTCTGAGGATCAC
SB-11-1	GTGGAGTGAGTGCTGTTCTTTCC
SB-11-2	TCTTCTGCTTGGGGAGCAG
SFTPC gene	
SC-1-1	CAGCAAGGAAGGCAGGCAC
SC-1-2	GAATGGATCTGGATAAGGAAACAGG
SC-2-1	TCCCTGTCCATCCATCGCATC
SC-2-2	GACAGTTTCCTATCGCCCATCC
SC-3-1	GAAAGAGGGAAGCGCATTGAG
SC-3-2	CTCTAGGTTGGGCACGGGAGTC
SC-4-1	CAGATTCTAGTATGACTCCCCTGC
SC-4-2	GAGGAACAGTGCTTTACAGGTGAC
SC-5-1	CTGGTGGCTTCTGACTCTAGCAC
SC-5-2	GAGTGGGAAGTACCGGTCTGTGAG



Supplementary Figure S1. Cellular effects of ABCA3-WT and ABCA3-R288K stably expressed in A549 cells. (A) ABCA3 mRNA levels analyzed by quantitative real time PCR ($p < 0.001$ for WT/mock and $p < 0.01$ for R288K/mock). (B) Western immunoblot analysis of HA-tagged ABCA3 in total cell lysates. β -actin was used as a loading control. (C) Ratio of upper/lower ABCA3 processing form. (D) Cell proliferation assay of A549 cells stably transfected with ABCA3-WT protein of R288K variant compared to mock control. XTT tetrazolium salt was used for absolute quantification. (E) Impact of stably transfection method on cell cytotoxicity. Therefore lactate dehydrogenase was quantified in cell supernatants. (F) Co-immunostaining of HA-tagged ABCA3 with the lysosomal (lamellar body) marker CD63. (G) Co-immunostaining of HA-tagged ABCA3 with the ER marker calnexin. Scale: 10 μ m. Merge images present the overlay from green and red fluorescence. All results were expressed as mean \pm SEM.

A**B**

Supplementary Figure S2. Stable expression of ABCA3-WT and ABCA3-R288K protein in HEK293 cells: (A) Relative mRNA levels of HEK293 stably expressing ABCA3-WT and ABCA3-R288K analyzed by quantitative real time PCR compared to mock control.

* $P < 0.05$. (B) Immunoblot analysis of ABCA3-WT and ABCA3-R288K stably expressed in HEK293 cells. β -actin was used as loading control.