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Quantifying ABCA3 deficiency and ABCA3 variant-specific response to hydroxychloroquine

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List of abbreviations

AT II cell: alveolar type II cell

NRDS: neonatal respiratory distress

PC: phosphatidylcholine

DPPC: dipalmitoyl-phosphatidylcholine

SP-A: surfactant protein A

SP-B: surfactant protein B

SFTPB: surfactant protein B

SP-C: surfactant protein C

SFTPC: surfactant protein C

ATP: adenosine triphosphate

ABCA3: ATP-binding cassette subfamily A member 3

NBD: nucleotide-binding domain

RD: regulatory domains

TMD: transmembrane domains

EL: extracellular loop

IH: intracellular helice

EH: exocytoplasmic helices

ILD: interstitial lung disease

chILD: interstitial lung disease in children

ER: endoplasmic reticulum

LB: lamellar body

MVB: multivesicular body

WT: wild type

SD: standard deviation

nSD: normalized standard deviation

CFTR (also known as ABCC7): cystic fibrosis transmembrane conductance regulator

FVC: forced vital capacity

HCS: high-content screening

HCQ: hydroxychloroquine

KLR: Kids Lung Register

List of publications

1. **Yang X**, Forstner M, Rapp CK, Rothenaigner I, Li Y, Hadian K, Griese M. ABCA3 Deficiency-Variant-Specific Response to Hydroxychloroquine. Int J Mol Sci. 2023 May 3;24(9):8179. doi: 10.3390/ijms24098179. PMID: 37175887; PMCID: PMC10179277.
2. **Yang X**, Rapp CK, Li Y, Forstner M, Griese M. Quantifying Functional Impairment of ABCA3 Variants Associated with Interstitial Lung Disease. Int J Mol Sci. 2023 Apr 20;24(8):7554. doi: 10.3390/ijms24087554. PMID: 37108718; PMCID: PMC10141231.
3. Li Y, Seidl E, Knoflach K, Gothe F, Forstner ME, Michel K, Pawlita I, Gesenhues F, Sattler F, **Yang X**, Kroener C, Reu-Hofer S, Ley-Zaporozhan J, Kammer B, Krüger-Stollfuß I, Dinkel J, Carlens J, Wetzke M, Moreno-Galdó A, Torrent-Vernetta A, Lange J, Krenke K, Rumman N, Mayell S, Sismanlar T, Aslan A, Regamey N, Proesmans M, Stehling F, Naehrlich L, Ayse K, Becker S, Koerner-Rettberg C, Plattner E, Manali ED, Papiris SA, Campo I, Kappler M, Schwerk N, Griese M. ABCA3-related interstitial lung disease beyond infancy. Thorax. 2023 Jun;78(6):587-595. doi: 10.1136/thorax-2022-219434. Epub 2023 Feb 20. PMID: 36808083.
4. Forstner M, Lin S, **Yang X**, Kinting S, Rothenaigner I, Schorpp K, Li Y, Hadian K, Griese M. High-Content Screening Identifies Cyclosporin A as a Novel ABCA3-Specific Molecular Corrector. Am J Respir Cell Mol Biol. 2022 Apr;66(4):382-390. doi: 10.1165/rcmb.2021-0223OC. PMID: 34936540.

Articles 1 and 2 are part of this cumulative dissertation.

Contribution to the publications

1.1 Contribution to paper I

Title: Quantifying Functional Impairment of *ABCA3* Variants Associated with Interstitial Lung Disease

Project conceptualization, **X.Y.** and M.G. **(equal leading)**

Methodology, **X.Y. (leading)** and M.G. (support for statistical method)

Software, **X.Y. (leading)** and C.K.R. (support for software of gene prediction)

Result validation, **X.Y. (leading)**, M.F. and M.G. (support for final validation)

Formal analysis, **X.Y. (leading)** and C.K.R. (support for gene prediction and analysis)

Investigation, **X.Y. (leading)**, Y.L. and M.F. (support for experiment protocols)

Project resources, **X.Y. (leading)** and M.G. (support for database access)

Data curation, **X.Y. (leading)** and Y.L. (support for partial data curation)

Writing—original draft preparation, **X.Y. (unique)**

Writing—review and editing, **X.Y.**, M.G. **(equal leading),** M.F. (support for review)

Visualization, **X.Y. (leading)**, M.F., M.G. and C.K.R. (support with suggestions)

Project administration, **X.Y.** and M.G. **(equal leading)**, M.F. (support)

1.2 Contribution to paper II

Title: ABCA3 Deficiency—Variant-Specific Response to Hydroxychloroquine

Project conceptualization, **X.Y.** and M.G. **(equal leading)**

Methodology, **X.Y. (leading)**, M.F. (support for HCS protocol), and M.G. (support for statistical method)

Software, **X.Y. (leading)**, C.K.R., I.R. and K.H. (support for HCS machine software)

Validation, **X.Y. (unique)**

Formal analysis, **X.Y. (unique)**

Investigation, **X.Y. (leading)**, I.R. (support for HCS machine software analysis), and Y.L. (support for experiment protocols)

Project resources, **X.Y. (leading)**, M.F. and M.G. (support for database access)

Data curation, **X.Y. (leading)**, C.K.R. and M.G. (support for review)

Writing—original draft preparation, **X.Y. (unique)**

Writing—review and editing, **X.Y.**, M.G. **(equal leading),** M.F., C.K.R. (support for review)

Visualization, **X.Y. (leading)**, M.F., M.G. and C.K.R. (support with suggestions)

Project administration, **X.Y.** and M.G. **(equal leading)**

2. Introduction

2.1 Key role of pulmonary surfactant in normal lungs

Pulmonary surfactant consists of about 90% lipids and 10% specific proteins by weight [1, 2], which is synthesized and then secreted via exocytosis by alveolar type II (AT II) cells [2]. After secreted into alveolar space, surfactant forms a monolayer with one or more closely functionally associated lipid bilayers in places at the alveolar air-liquid interface [3] (Figure 1). Pulmonary surfactant reduces the alveolar surface tension when the alveolar surface is lowered to a minimum at the end of expiration, which makes it a crucial modulator for normal breathing [3, 4]. After performing its physiological functions, most of the surfactant is recycled via endocytosis, and part of the remainder is taken up by the alveolar macrophages, both of which are integrated into lamellar bodies locating within alveolar type II cells [3]. A lack of surfactant causes gas exchange disturbance and affected individual may suffer from neonatal respiratory distress (NRDS) or even neonatal death [1]. Surfactant dysfunction is also playing an important role in adult respiratory diseases, which could be explained by surfactant's involvement in several defense mechanisms of lungs, e.g. host defense against infection and inflammation [5].

The major surfactant lipids are phospholipids (90 - 95% by weight) [6]. Among the phospholipids components, phosphatidylcholine (PC) and its active species dipalmitoyl-PC (DPPC) are the most prevalent key components, accounting for about 80% of the total phospholipids [1]. Generally, DPPC is tightly packed, which can effectively eliminate the direct air-liquid contact and reduce the surface tension when the alveolar surface area is reduced [7]. DPPC is not only produced by *de novo* synthesis, but also by recycled from pulmonary surfactant [1, 8]. It has been estimated in 3-day-old rabbits that the efficiency of recycling surfactant PC could be as high as 95%, which is only about 55% in

the adult rat lungs [9-11]. It is helpful to better understand metabolism pathway of PC and/or DPPC, when estimating potential surfactant dysfunction in specific related lung diseases.

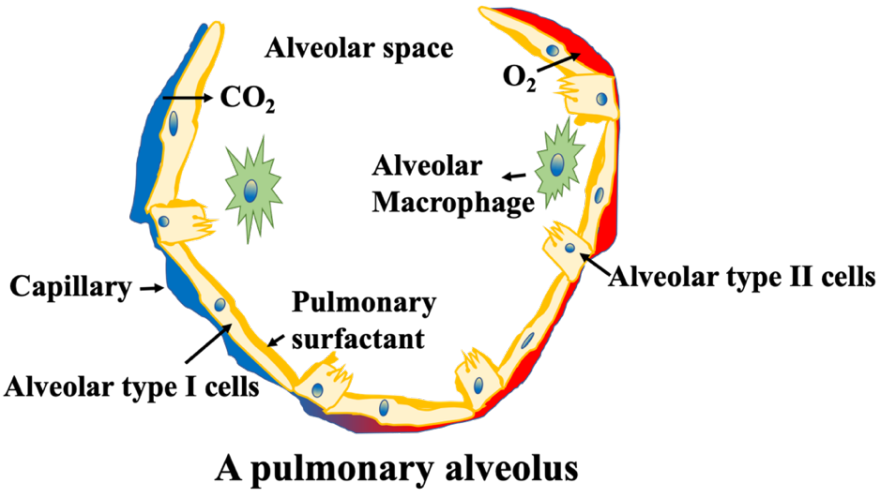

Figure 1: Pictogram of structure of a pulmonary alveolus.

2.2 Surfactant-related interstitial lung diseases in children

Interstitial lung diseases (ILD) in children is a broad spectrum of chronic lung conditions frequently related to inflammation and sometimes to pulmonary fibrosis, characterized by diffuse infiltrates, restrictive functional defect and gas exchange disorders [12-14]. Patients with ILD often develop respiratory symptoms, such as dry cough, shortness of breath during physical exercising or even at rest in severe cases [14, 15]. Physical examination commonly reveals increased respiratory rate at rest and rales or crackles in the basal lungs segments [14, 15]. To note, a spectrum of interstitial pulmonary dysfunction occurs mainly in children, covering genetic disorders of surfactant metabolism [15, 16]. The majority of patients with surfactant dysfunctions are diagnosed with gene analysis. Pathogenic adenosine triphosphate (ATP)-binding cassette subfamily A member 3 (*ABCA3*) variants, *SFTPB* variants, and *SFTPC* variants have been found to cause surfactant dysfunctions, which play key roles in the pathogenesis of lung diseases [15, 17]. Generally, mutant SFTPB is related to severe respiratory distress in the first month of life

[18]. Mutant SPTPC is more frequently linked to ILD in children beyond the neonatal period, or in adults, whereas patients with mutant ABCA3 have various clinical manifestations [18].

2.3 ABCA3 deficiency: a monogenetic cause for surfactant-related interstitial lung diseases

To date, there are 588 *ABCA3* variants reported in ClinVar (444 of 588 *ABCA3* variants are reported in gnomAD v2.1.1). Among the ClinVar variants, only 222 *ABCA3* variants are identified, with 52 pathogenic or likely pathogenic *ABCA3* variants and 170 benign or likely benign *ABCA3* variants. The other 366 ClinVar *ABCA3* variants are categorized as variants of uncertain significance (Data resource from: https://gnomad.broadinstitute.org/gene/ENSG00000167972). ABCA3 deficiency is a common monogenetic cause for pulmonary surfactant disorders [15, 19, 20]. Increasing evidence indicates respiratory conditions of patients with *ABCA3* variants depend on the level of ABCA3 dysfunctions, with a broad range from neonatal respiratory distress and deaths in full-term infants, progressive ILD in children or adults, and free of respiratory symptoms [19, 21]. In lung histology, characteristic abnormal lamellar bodies could be detected on electron microscopic level [22, 23]. However, there are variable unspecific patterns on light microscopic level [24]. Chest high-resolution CT images might demonstrate a sustained ground glass opacities pattern [24], whereas typical features of fibrosis were with interindividual heterogeneity and progression with age [24]. A retrospective study of 44 ABCA3-deficient patients surviving more than 1 year after birth indicated clearly progressive ILD in this cohort, with a decline of 1.1% per year in forced vital capacity (FVC) % predicted [24]. Apart from lung transplantations, currently no curative treatment exists for ABCA3 deficiency [19].

2.4 Biogenesis and function of ABCA3

2.4.1 Structure of ABCA3 protein

ABCA3 is a member of the transmembrane ABC superfamily of transporters [25, 26]. As illustrated in Figure 2, it consists of 2 nucleotide binding domains (NBDs), 2 regulatory domains (RDs) and 2 transmembrane domains (TMDs) with 6 TM helices respectively [27]. The TMDs fold discretely, with two large extracellular loops (EL1 and EL2) and four intracellular helices (IHs), i.e. IH1 before TM1 and IH3 before TM7, IH2 between TM2 to TM3 and IH4 between TM8 to TM9, and four exocytoplasmic helices (EHs), i.e. EH1 and EH2 inserted between TM5 to TM6, EH3 and EH4 inserted between TM11 to TM12 [27]. ABCA3 protein localizes at the external membranes of lamellar bodies (LBs), which are key compartments for the pulmonary surfactant storage in AT II [28]. Normal ABCA3 protein is essential for LBs biogenesis and surfactant-lipid transportation with its ATP hydrolysis activity [28].

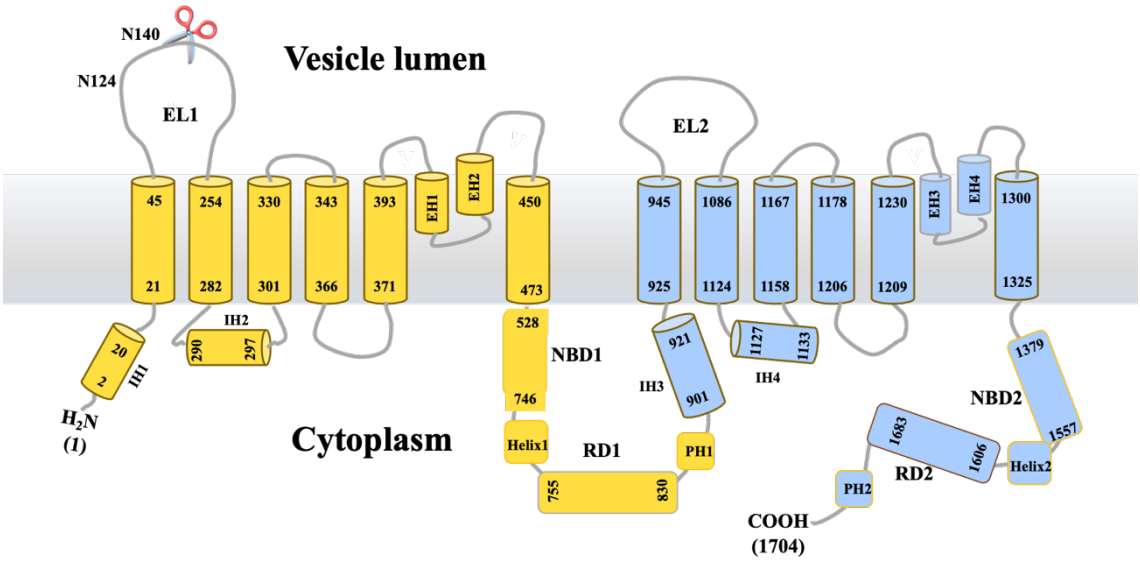

Figure 2: Topology model of ABCA3 protein. EL1: the first extracellular loop; EL2: the second extracellular loop. RD1: regulatory domain 1; RD2: regulatory domain 2. IH1: intracellular helice 1; IH2: intracellular helice 2; IH3: intracellular helice 3; IH4: intracellular helice 4. EH1: exocytoplasmic helice 1; EH2: exocytoplasmic helice 2; EH3: exocytoplasmic helice 3; EH4: exocytoplasmic helice 4. NBD 1: nucleotide binding domain 1; NBD 2: nucleotide binding domain 2. The scissors icon indicates the amino acid site for proteolytic cleavage of ABCA3 protein. N124 and N140 are at amino acid sites for N-glycosylation of ABCA3 protein.

2.4.2 Intracellular trafficking and function of ABCA3

As illustrated in Figure 3, after correct transcription, ABCA3 is translated in the endoplasmic reticulum (ER) and then is routed to Golgi apparatus for N-glycosylation, which is necessary for the stability of ABCA3 protein [26, 29]. It has been demonstrated that glycosylation at amino acid site N124 and N140 is indispensable for correct trafficking of ABCA3, and the ER has a checking mechanism for the N-glycosylation process [29]. With correct N-glycosylation, ABCA3 is trafficked to multivesicular bodies, and then localizing the external membranes of LBs [26, 30]. Within these lysosome-related compartments, ABCA3 is involved in LB biogenesis and transports those surfactant lipids both from *de novo* synthesis and from surfactant recycling into LBs, using the energy from ATP-hydrolysis activity [1, 28]. After the lipoprotein complex of pulmonary surfactant is secreted into the alveolar space, ABCA3 is either reused in the existing LBs or will undergo proteolytic cleavage in lysosomes [30-34]. It has been found that cleavage

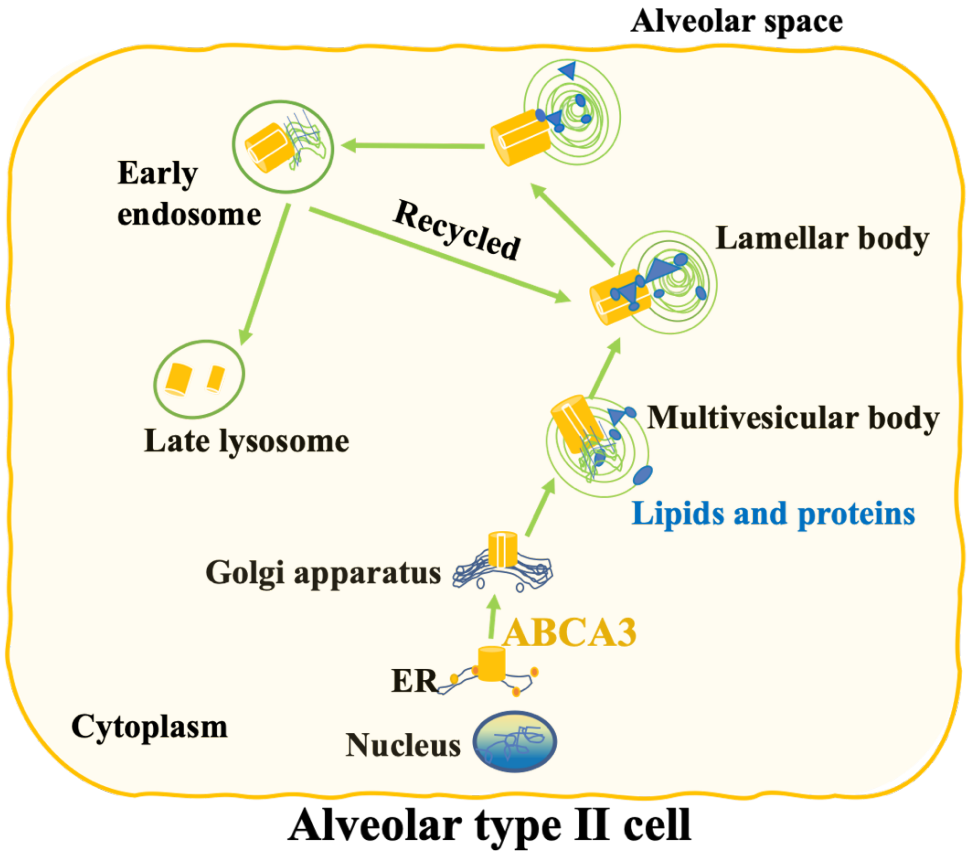

Figure 3: Intracellular trafficking of ABCA3 within an alveolar type II cell. ER: endoplasmic reticulum.

of ABCA3 occurs after K174 at the first extracellular loop of ABCA3 [33]. The removal of this about 20 kDa segment from the full-length ABCA3 protein profoundly alters the structure of primary ABCA3 and yield a cleaved product, which is likely to regulate ABCA3 levels, though specific mechanisms need to be further clarified [33].

2.4.3 Classification of *ABCA3* variants based on genotypes

The genotype of *ABCA3* variant and its associated dysfunction strongly predicts the outcome of children with ABCA3 deficiency [24, 35]. Nonsense and frameshift variants were defined as "null" variants, which commonly caused a complete loss of ABCA3 function [35]. The majority of patients with biallelic complete loss-of-function variants develop respiratory distress or die in infancy, whereas patients with "hypomorphic" variants, such as in-frame insertions or missense variants, may possibly survive with chronic ILD, or with mild or little respiratory symptoms [24, 35, 36]. Despite a genotype–phenotype pattern exists, the wide range of clinical presentations makes it very challenging to clearly distinguish the residual function of ABCA3 protein, which might help support clinical management according to the patients' disease prognosis. Therefore, it is essential to gain comprehensive data from *in vitro* experiments on *ABCA3* variants.

2.4.4 Classification of *ABCA3* variants based on *in vitro assays*

At present, it is still not possible to evaluate the effects of *ABCA3* variants based exclusively on in silico prediction and structure analysis [27, 37]. Deep biological knowledge of *ABCA3* variants is relatively poor and mainly obtained from methodologically heterogeneous *in vitro* studies on cellular level [22, 23, 26, 28, 29, 31, 34, 38-50]. ABCA3 variants have been previously grouped into trafficking mutations, i.e., resulting in intracellular mis-localization and dysfunction of ABCA3 protein, and functional mutations, i.e., having normal intracellular ABCA3 protein trafficking but lipid pumping

impairment [31, 38-40, 48]. However, this level of classifications might lose too many details from different trafficking and functional assays to come to precise interpretations [37]. It is essential to come up with a quantitative method to compile results from different assays, and to carry out a quantitative correlation analysis with data from *in vitro* experiment and data from clinical observations, so as to better interpret the effects of *ABCA3* variants and to predict patient's outcomes more accurately, with a view to implement precise treatments [21, 35, 38, 40].

Therefore, in this study, we first characterized and quantified overall *in vitro* dysfunction of ABCA3 variants by assessing their intracellular trafficking and pumping activity [37]. Then a definition was proposed to describe the deviation of trafficking and pumping for ABCA3 variants, i.e., normal, impaired and defective, respectively (Figure 4) [37].

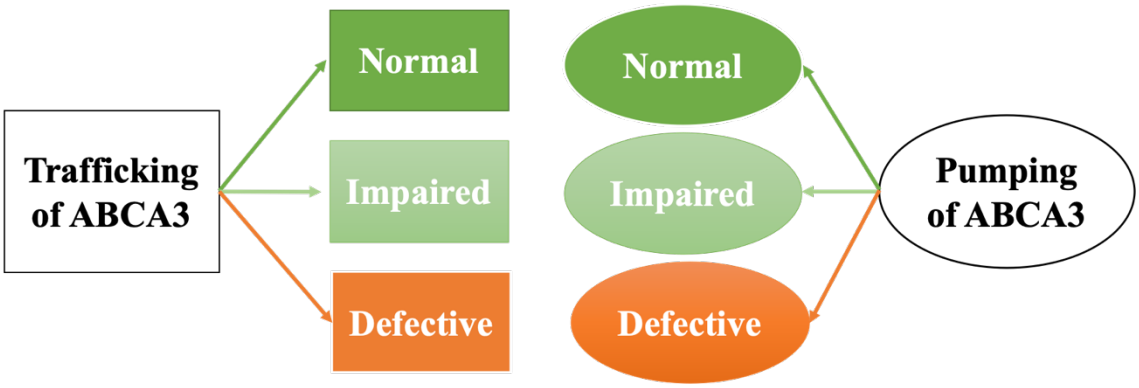

Figure 4: Illustration of ABCA3 dysfunction based on quantitative readouts from trafficking and pumping *in vitro* assays.

For a further *in vivo* and *in vitro* correlation analysis, quantitative *in vitro* dysfunction of ABCA3 variants from different studies were correlated to the outcomes of patients carrying those specific variants [37]. The sum of the quantitative trafficking and pumping *in vitro* was helpful to predict a clinical outcome [37]. An approximately 50% loss of function *in vitro* was a threshold for considerable morbidity and mortality [37].

2.5 Therapeutic strategy for ABCA3 deficiency related lung diseases

With the structural similarities of ABCA3 and ABCC7 (see the list of abbreviations at page 7), possible approaches to treat ABCA3-deficiency have been modeled in analogy to the approaches to treat cystic fibrosis caused by ABCC7 variants [51-53]. It has been proven in *in vitro* studies that several mutant ABCA3 protein could be rescued by low temperature and small molecules ivacaftor and genistein, or Cyclosporin A (CsA) [38, 39, 54]. However, to our knowledge, apart from lung transplantations, currently there is no cure available for patients with ABCA3 deficiency related lung diseases.

2.5.1 Hydroxychloroquine for patients with ABCA3 deficiency

Apart from steroids, hydroxychloroquine (HCQ) is frequently used to treat children with interstitial lung diseases empirically, either as a single treatment or combined with other medicines [55-57]. Currently, the respiratory outcomes of patients with ABCA3 deficiency under HCQ therapy are inconclusive, varying from remarkable respiratory improvements, or partial improvement with remaining chronic ILD, or no response with sustained respiratory deteriorations [55, 56]. As data from randomized controlled clinical trials are unlikely to be available in the near future, it is of certain value to collect retrospective clinical data of patients with *ABCA3* variants and to correlate to the *in vitro* response of these variants [36].

2.5.2 Effects of hydroxychloroquine on intracellular trafficking and pumping of ABCA3

As a weak base, HCQ has a well-known lysosomotropism activity [58-60]. As mentioned above, ABCA3 protein locates at the outer specific membranes of LBs, which are acidic lysosome related compartments [26, 31]. Currently, it has not been extensively studied whether HCQ has effects on the intracellular biogenesis of ABCA3, or on the lipid pumping activities of ABCA3 in appropriate *in vitro* models [36].

In this study, we first retrospectively reviewed the individual clinical courses of 39 patients with ABCA3 deficiency, who received HCQ therapy exclusively or in combination with other small molecule agents [36]. Then we explored the *in vitro* effect of HCQ with different concentrations on sixteen mutant ABCA3-HA tagged transfected A549 cells [36]. At the last step, we analyzed the correlation of *in vivo* data and *in vitro* results [36]. The respiratory responses to HCQ treatment were linked to the *ABCA3* variants in patients [36]. A mean value of ABCA3 positive vesicle volume > 60% of the wild type volume may predict a concentration-dependent response to HCQ *in vitro* [36]. There was a moderate correlation between the *in vitro* and *in vivo* responses to HCQ [36].

3. Summary (in Englisch)

Pathogenic *ABCA3* variants are currently known as a common monogenetic cause for surfactant dysfunction [15, 19, 20]. The quantitative ABCA3 overall function *in vitro* allows for in-depth variant characterization and correlation analysis to *in vivo* observation, which possibly supports treatment decisions in patients [37]. More detailed biological data on more variants will be necessary to empower the prediction for the broad spectrum of *ABCA3* variants in asymptomatic carriers or patients.

At present, there is no proven cure drug for patients with ILD caused by ABCA3 deficiency [19]. As a common empirical treatment for chILD, HCQ showed variant-specific effects on ABCA3 *in vitro*, with supportive evidence from retrospective clinical data on the patients with ABCA3 deficiency and under HCQ treatment, though various constrains existed [36]. Prospective and randomized controlled trials in patients with ABCA3 deficiency are necessary in the future, as well as the establishment of advanced *in vitro* models to better develop personalized treatments.

4. Zusammenfassung (deutsch)

Krankheitsauslösende *ABCA3* Varianten sind der am häufigsten beschriebene Grund für monogenetisch bedingte Lungenerkrankungen des Surfactantsystems im Kindes- und Jugendalter. In dieser Arbeit wurde durch eine *in-vitro* Quantifizierung der Lipid-Transporterfunktion von ABCA3 eine detaillierte Charakterisierung pathogener *ABCA3* Varianten vorgenommen. Diese Daten wurden zu *in-vivo* Beobachtungen an Patienten korreliert und eine detailliertere Genotyp-Phänotyp-Korrelation herausgearbeitet. Obwohl hier eine relativ große Anzahl an Varianten charakterisiert wurde, sind weitere biologische Daten von weiteren Varianten notwendig, um das breite klinische Spektrum von *ABCA3* Varianten bei Patienten und asymptomatisch Mutationsträgern zu verstehen und deren Auswirkungen sicher zu prognostizieren.

Bis heute gibt es keine etablierte medikamentöse Therapie der ABCA3-assoziierten Lungenerkrankungen. In dieser Arbeit wurde der Zusammenhang zwischen retrospektiv erhobenen klinischen Daten von molekular charakterisierten Patienten mit ABCA3-Defizienz die mit Hydroxochloroquine (HCQ) behandelt wurden und *in-vitro* HCQ-Exposition von ABCA3 Varianten untersucht. Es fand sich ein lockerer Zusammenhang. Vielfältige Störfaktoren konnten nicht kontrolliert werden. Zukünftige, am besten prospektive Untersuchungen an Patienten mit ABCA3-Defizienz sind wünschenswert, um personalisierte Behandlungsoptionen basierend auf Zellmodellen weiter zu entwickeln.

5. Paper I

Title of article： Quantifying Functional Impairment of *ABCA3* Variants Associated with Interstitial Lung Disease

Title of journal：International Journal of Molecular Science

Year: 2023

Volume: 24

Pages: 7554

Authors: Xiaohua Yang, Christina K. Rapp, Yang Li, Maria Forstner, Matthias Griese.

PMID: 37108718; PMCID: PMC10141231. DOI: 10.3390/ijms24087554

6. Paper II

Title of article： ABCA3 deficiency – Variant-Specific Response to Hydroxychloroquine

Title of journal： International Journal of Molecular Science

Year: 2023

Volume: 24

Pages: 8179

Authors: Xiaohua Yang, Maria Forstner, Christina K Rapp, Ina Rothenaigner, Yang Li, Kamyar Hadian, Matthias Griese

PMID: 37175887; PMCID: PMC10179277. DOI: 10.3390/ijms24098179.

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