DISSERTATION
DER FAKULTÄT FÜR BIOLOGIE
DER LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Defining Childhood Interstitial Lung Disease on the Molecular Level: Insights Into Known and Novel Genetic Entities Predisposing to Fibrosis

vorgelegt von

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München, 2022

Diese Dissertation wurde unter der Leitung von Prof. Dr. med. Matthias Griese am Dr. von Haunerschen Kinderspital der Ludwig-Maximilians-Universität München (LMU) angefertigt und von Prof. Dr. rer. nat. Christof Osman, Bereich Zell- und Entwicklungsbiologie der LMU, vor der Fakultät für Biologie vertreten.

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Tag der Abgabe: 03.02.2022
Tag der mündlichen Prüfung: 14.07.2022

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Summary

Childhood interstitial lung diseases (chILD) are a heterogenous group of rare disorders affecting the pulmonary connective tissue scaffold and its associated structures. A subset of chILD cases develops pulmonary fibrosis (PF), a severe pathological lung remodeling process that is potentially life-threatening. Early identification of cases at high risk for developing PF is therefore crucial but remains a challenge in current clinical practice. As rare diseases are often hereditary monogenic traits, we used extended genetic analysis to study the molecular nature of chILD with PF features in children and adolescents. We provide an in-depth clinical characterization of a previously known but underexplored genetic disorder with PF, Hermansky-Pudlak syndrome type 2. In addition, novel genetic entities are presented that are linked to unique multisystem disorders associated with childhood PF. We relate bi-allelic variants in cytosolic phenylalanyl-tRNA synthetase genes (*FARSA, FARSB*) to a spectrum of multiorgan dysfunctions and continue to functionally analyze the role of the affected enzyme, FARS1, in aminoacylation and protein biosynthesis. We further identify a novel inborn error of immunity based on deficiency in ZNFX1, a viral nucleic acid sensor, and investigate its effects on immune regulation, viral clearance, and chronic inflammation. Lastly, this work offers perspectives on personalized treatment of chILD as a potential future therapy on the horizon.

1. Introduction

1.1 Rare diseases in respiratory medicine

Rare diseases represent individually sporadic conditions that collectively have a significant impact on global health and its socio-economic costs[1-3]. Within the European Union, a rare disease is defined by a prevalence of ≤ 5 in 10,000 individuals[4]. Given a cumulative global point prevalence estimate of ~3.5-5.9%, up to ~30 million people in Europe and ~446 million people worldwide could be affected by a rare disease at any point in time[5].

Rare diseases are frequently congenital, hereditary monogenic traits[5,6]. In the field of respiratory medicine, monogenic diseases can manifest as disorders affecting the airways, the pulmonary parenchyma, or pulmonary vasculature[7]. They also occur as sleep disorders or in the form of systemic diseases with lung involvement[7]. In children, specific rare lung diseases are often difficult to recognize and diagnose due to their largely unknown clinical presentation, their broad phenotypic overlap with other conditions, and additional common comorbidities such as respiratory infections[8,9].

1.2 Interstitial lung diseases

Among the rare pulmonary disorders, interstitial lung diseases (ILD) represent a heterogeneous group of disorders related to the interstitium, the connective tissue scaffold of the lung[9]. In the periphery of the lungs, ILDs affect the alveolar epithelium, the capillary vessel endothelium, and the tissues between these structures[9,10]. More centrally, they can involve peribronchial and peribronchiolar tissues including airways, as secondary in the disease process[9,10].

Different ILD subgroups can be distinguished based on the nature of the underlying condition (Fig.1). Major categories include ILDs manifesting as a consequence of systemic diseases such as e.g. connective tissue disease (CTD) or sarcoidosis[11]. Other ILDs can be caused by specific environmental exposures such as hypersensitivity pneumonitis (HP), or certain drugs[11]. Many other forms are idiopathic[11]. In infancy and childhood, more prevalent conditions include various disorders of the developing lung, surfactant dysfunction disorders, and particular conditions of unclear etiology restricted to infancy[12,13].

As indicated in red, a part of the subgroups develops progressive fibrosing ILD. Fibrosis represents a tissue remodeling process, in which original lung parenchyma is replaced by excessive amounts of extracellular matrix (ECM) leading to scar formation and irreversible organ dysfunction[14,15]. These changes are considered the end result of a chronic inflammatory

reaction triggered by different organ-damaging external and internal stimuli, e.g. chemicals, radiation, allergens, or certain genetic variants that intrinsically affect pulmonary cell integrity[16].

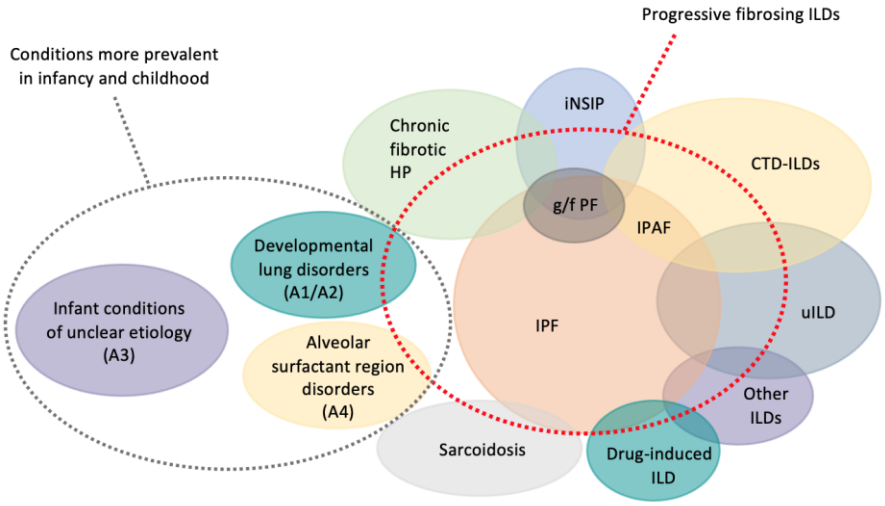

Fig. 1. Overview of ILD subgroups with potentially fibrosing phenotype in childhood and adulthood. Conditions more prevalent in infancy and childhood include developmental lung disorders, surfactant dysfunction disorders, and particular infant conditions of unclear etiology (left)[12,13]. Conditions more prevalent in adulthood comprise hypersensitivity pneumonitis (HP), idiopathic nonspecific interstitial pneumonia (iNSIP), connective tissue disease (CTD)-ILDs, sarcoidosis, drug-induced ILD, and cases of unclassifiable ILD (uILD)[11]. The group of CTD-ILDs include rheumatoid arthritis-associated ILD, systemic sclerosis-associated ILD, mixed CTD-associated ILD and other autoimmune ILDs[11]. Other ILDs include exposure-related ILDs (asbestosis and silicosis), non-idiopathic pulmonary fibrosis (IPF), idiopathic interstitial pneumonias (desquamative interstitial pneumonia, etc.), and others (right)[11]. g/f PF: genetic and/or familial pulmonary fibrosis; IPAF: interstitial pneumonia with autoimmune features. Modified from *European Respiratory Review*[11]. Childhood classifications according to *Orphanet Journal of Rare Diseases*[9].

1.3 Fibrosing lung disease in childhood and adulthood

One of the most common progressively fibrosing interstitial lung disease in adults is idiopathic pulmonary fibrosis (IPF)[17]. Overall IPF prevalence estimates range between 1-63 per 100,000 population and are substantially increased for higher age categories, with a peak in the age class of ≤ 75 years[18]. Clinically, patients present with symptoms of increasing dyspnea caused by fibrotic processes progressively distorting lung architecture[19,20] (Fig.2). These changes are reflected in typical radiologic imaging showing interlobular septal thickening, formation of

honeycomb cysts in predominantly peripheral distribution, and traction bronchiectasis[21]. Histologically, IPF is characterized by sites of normal lung tissue interspersed with patchy areas of active fibroblast proliferation called fibroblastic foci[21,22]. Lung function shows restrictive physiology[21]. Taken together, these findings are indicative of usual interstitial pneumonia (UIP), the hallmark pattern of IPF[21]. IPF has a poor prognosis with a median survival of 2-5 years after diagnosis, comparable to different forms of cancer[23-25].

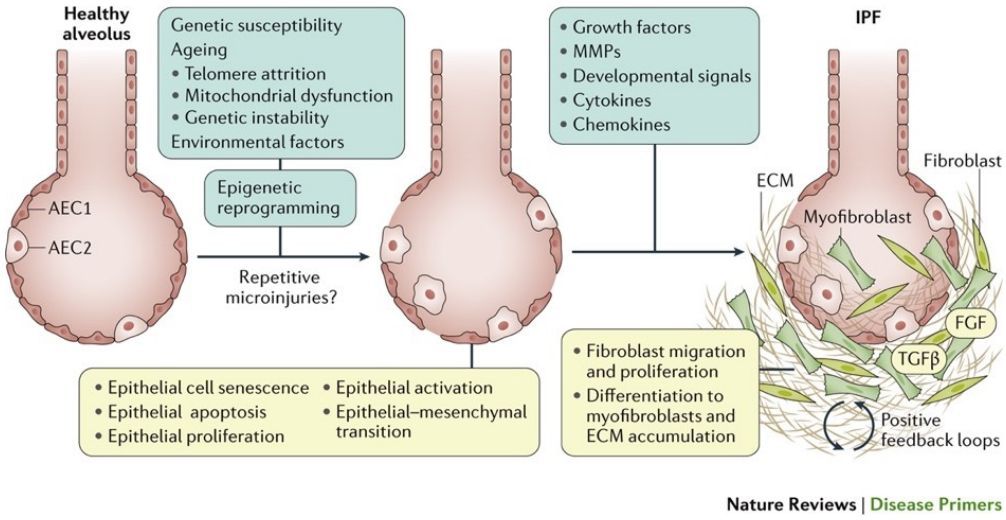

Fig. 2. Schematic model of IPF pathophysiology. Individual genetics, aging, specific environmental factors, and epigenetic reprogramming determine alveolar epithelial cell integrity and its susceptibility to external insults[15,26]. Presumed repetitive microinjuries may exacerbate or lead to alveolar epithelial cell apoptosis, subsequently increased epithelial proliferation, and epithelial-mesenchymal transition events[15]. Fibroblasts are recruited and differentiate into myofibroblasts, which are the main drivers of extracellular matrix (ECM) deposition and lung remodeling[15,27]. In a positive feedback loop, immune cells, cytokines, and a plethora of additional molecular factors orchestrate and maintain what is considered an "aberrant wound healing process"[15,26]. AEC1: alveolar epithelial cell type 1; AEC2: alveolar epithelial cell type 2; MMPs: matrix metalloproteinases; ECM: extracellular matrix. Reprinted from *Nature Reviews Disease Primers*[15], with permission from Springer Nature.

The pathophysiology of IPF is currently incompletely understood. It is hypothesized that a complex interplay between genetics, aging, and environmental factors causes initial disease onset[28]. A number of genetic variants that can cause spontaneous disease or predispose to disease have been identified. Among them are rare variants in genes linked to surfactant metabolism (*SFTPA1, SFTPA2, SFTPC, ABCA3*) and telomere maintenance *(TERC*, *TERT, TINF2, DKC1, RTEL1, PARN)*[29]. Common polymorphisms associated with IPF are connected to gene functions in airway mucin production *(MUC5B, MUC2)*, cell-cell adhesion *(DSP,*

DPP9), telomere maintenance *(TERT, OBFC1)*, and cell cycle regulation *(KIF15, MAD1L1, CDKN1A, TP53)*[29].

The contribution of the immune system to IPF pathology has been controversially debated[30,31]. While initial concepts viewed immune cells as the primary mediators of fibroblasts activation and recruitment in response to epithelial damage, new research suggests that fibrosis occurs as an independent process that is, however, modulated and shaped by immune cell activity[31]. Genetic variants in toll-like receptor signaling (*TOLLIP, TLR3, ATP11A*) and cytokine/growth factor signaling *(IL1RN, IL8, IL4, TGFB1)* linked to IPF highlight the important role of the immune system in disease pathogenesis[29].

Fibrosing lung disease also occurs in childhood; however, across the childhood interstitial lung disease (chILD) spectrum it is an extremely rare condition, which remains poorly characterized[32]. Compared to adult forms of lung fibrosis such as IPF, the UIP pattern typical for IPF is almost never observed[32,33]. Instead, the predominant interstitial pneumonia pattern in children is characterized by more inflammatory cell infiltration, less fibroblast recruitment and ECM deposition, and a generally uniform appearance across the lungs[32]. Whether childhood forms of ILD and fibrosis could develop into adult forms and whether fibrosis in the developing and aging lung is governed by the same mechanisms is currently discussed[32,33].

1.4 Genetic architecture of disease

In pediatric diseases, especially in those with extremely rare, strong and early-onset clinical picture, the inherited component to disease is typically much more prominent than in diseases that manifest later in life[34]. On a spectrum that describes frequency and effect of individual genetic variants, these cases are classically categorized as monogenic diseases[35] (Fig.3). In monogenic diseases, single gene variations can cause spontaneous disease onset. To date, numerous single gene-disease associations are reported[36]. However, in clinical practice, many patients also remain undiagnosed, even though a genetic etiology is suspected.

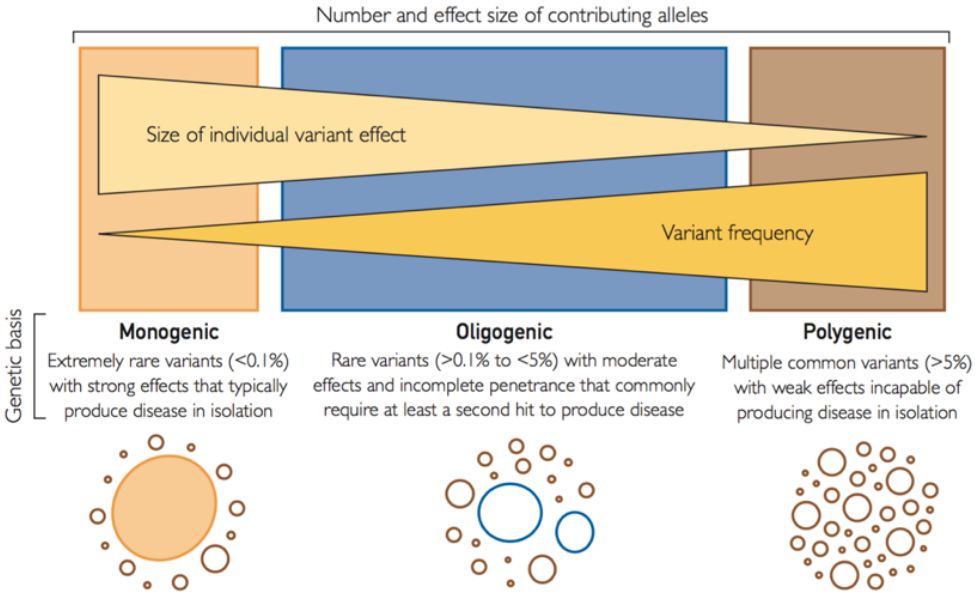

Fig. 3. Genetic architecture of disease model. Disease-contributing alleles exist on a spectrum that is determined by their effect and frequency. It reaches from a monogenic pattern, in which extremely rare variants can cause strong phenotypes in isolation (left) to a polygenic pattern, in which multiple common variants with weak individual effects collectively work to produce disease, often in combination with environmental factors (right). The oligogenic pattern represents a mixture of the two extremes, in which few rare variants with moderate effect produce disease (middle). Adapted from *Mayo Clinic Proceedings*[35], with permission from Elsevier.

1.5 Expanding the molecular understanding of fibrosing lung disease in childhood

Different experimental approaches are employed to identify genetic variations that might cause or predispose to disease. In children for which a monogenic cause is currently unknown but highly likely, genome-based next generation sequencing (NGS) approaches are typically used to pinpoint potential candidate genes[37]. It is estimated that ~85% or more of the variants linked to monogenic disorders can be found in protein-coding regions, which comprise only ~2% of the total genomic code[38,39]. Therefore, whole exome sequencing (WES), i.e. the sequencing of exons and exon-flanking regions of all protein-coding genes, has emerged as a particularly useful and cost-effective tool in clinical diagnostics and research[40]. Throughout this work, we used WES to explore the underlying genetics of children with fibrosing lung disease of unknown etiology.

To this end, we recruited patients from the European Management Platform for Childhood Interstitial Lung Diseases (chILD-EU), an international clinical registry with biobank that supports diagnostics, research, and therapeutic care for children with rare

interstitial lung diseases[41]. For this study, we selected a cohort with a particularly high disease burden, children with unique multisystemic clinical symptoms in addition to their fibrosing lung disease, and studied both subgroups of defined and unknown genetic etiology (Fig.4).

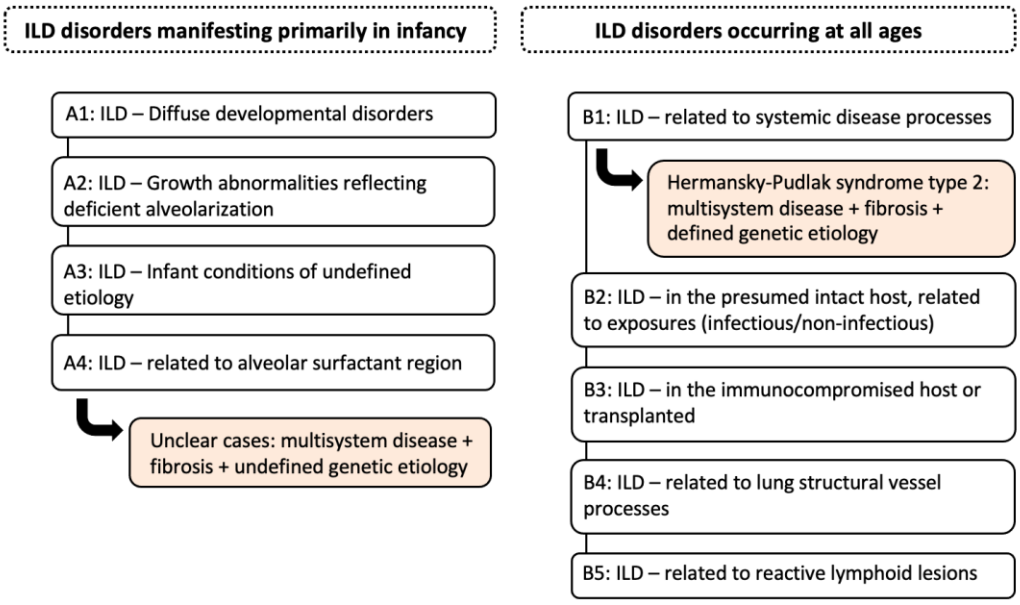

Fig. 4. Overview of current chILD-EU classification and patient cohort recruitment. The child-EU registry was searched for cases of multisystem diseases with histologic evidence of lung fibrosis. The obtained cases include, among others, Hermansky-Pudlak syndrome type 2 (HPS-2), a cohort of defined genetic etiology but currently little phenotypic characterization. HPS-2 patients were retrieved from the subcategory of ILD patients with systemic disease processes. Additional cases with multisystem disease, fibrosis, but undefined genetic etiology were recruited from the subcategory of patients with ILD related to the alveolar surfactant region. Those genetically unclear cases were further examined using WES. Classification and schematic reproduced from *Orphanet Journal of Rare Diseases*[9].

1.6 Aim of the thesis

The overarching goal of the dissertation project was to characterize potentially fibrosing childhood interstitial lung disease (chILD), as this cohort has not yet been described to a large extent. Progress in this field will enable rapid diagnostics, mechanistic understanding, and ultimately treatment for children with this rare condition. Based on clinical and biological data from a large European registry, we selected a genetically defined but currently little characterized chILD entity (Hermansky-Pudlak syndrome type 2) and explored its clinical phenotype in detail. Where necessary, comprehensive genetic analyses were performed to identify novel gene candidates defining previously unknown entities of disease with suspected monogenic inheritance pattern and investigate potential mechanistic links to disease.

2. Results

2.1 Article 1

Title	Hermansky-Pudlak syndrome type 2 manifests with fibrosing lung disease early in childhood
Authors	Hengst, M., Naehrlich, L., Mahavadi, P., Grosse-Onnebrink, J., Terheggen-Lagro, S., Skanke, L. H., **Schuch, L. A.**, Brasch, F., Guenther, A., Reu, S., Ley-Zaporozhan, J. & Griese, M.
Journal	*Orphanet J Rare Dis* 13, 42
Year	2018
DOI	**https://doi.org/10.1186/s13023-018-0780-z**

2.2 Article 2

Title	Bi-allelic Mutations in Phe-tRNA Synthetase Associated with a Multi-system Pulmonary Disease Support Non-translational Function
Authors	Xu, Z.*, Lo, W. S.*, Beck, D. B.*, **Schuch, L. A.***, Olahova, M., Kopajtich, R., Chong, Y. E., Alston, C. L., Seidl, E., Zhai, L., Lau, C. F., Timchak, D., LeDuc, C. A., Borczuk, A. C., Teich, A. F., Juusola, J., Sofeso, C., Müller, C., Pierre, G., Hilliard, T., Turnpenny, P. D., Wagner, M., Kappler, M., Brasch, F., Bouffard, J. P., Nangle, L. A., Yang, X. L., Zhang, M., Taylor, R. W., Prokisch, H., Griese, M., Chung, W. K. & Schimmel, P. *shared first authorship
Journal	*Am J Hum Genet* 103, 100-114
Year	2018
DOI	**https://doi.org/10.1016/j.ajhg.2018.06.006**

2.3 Article 3

Title	FARS1-related disorders caused by bi-allelic mutations in cytosolic phenylalanyl-tRNA synthetase genes: Look beyond the lungs!
Authors	**Schuch, L. A.***, Forstner, M.*, Rapp, C. K., Li, Y., Smith, D. E. C., Mendes, M. I., Delhommel, F., Sattler, M., Emiralioglu, N., Taskiran, E. Z., Orhan, D., Kiper, N., Rohlfs, M., Jeske, T., Hastreiter, M., Gerstlauer, M., Torrent-Vernetta, A., Moreno-Galdo, A., Kammer, B., Brasch, F., Reu-Hofer, S. & Griese, M. *shared first authorship
Journal	*Clin Genet* 99, 789-801
Year	2021
DOI	**https://doi.org/10.1111/cge.13943**

2.4 Article 4

Title	Multisystem inflammation and susceptibility to viral infections in human ZNFX1 deficiency
Authors	Vavassori, S., Chou, J., Faletti, L. E., Haunerdinger, V., Opitz, L., Joset, P., Fraser, C. J., Prader, S., Gao, X., **Schuch, L. A.**, Wagner, M., Hoefele, J., Maccari, M. E., Zhu, Y., Elakis, G., Gabbett, M. T., Forstner, M., Omran, H., Kaiser, T., Kessler, C., Olbrich, H., Frosk, P., Almutairi, A., Platt, C. D., Elkins, M., Weeks, S., Rubin, T., Planas, R., Marchetti, T., Koovely, D., Klambt, V., Soliman, N. A., von Hardenberg, S., Klemann, C., Baumann, U., Lenz, D., Klein-Franke, A., Schwemmle, M., Huber, M., Sturm, E., Hartleif, S., Haffner, K., Gimpel, C., Brotschi, B., Laube, G., Gungor, T., Buckley, M. F., Kottke, R., Staufner, C., Hildebrandt, F., Reu-Hofer, S., Moll, S., Weber, A., Kaur, H., Ehl, S., Hiller, S., Geha, R., Roscioli, T., Griese, M. & Pachlopnik Schmid, J.
Journal	*J Allergy Clin Immunol* 148, 381-393
Year	2021
DOI	**https://doi.org/10.1016/j.jaci.2021.03.045**

3. Discussion

Childhood interstitial lung diseases (chILD) are a heterogenous group of conditions that differ in etiology, pathology, prognosis, and treatment[42]. Among those affected, a subset develops pulmonary fibrosis (PF)[32]. PF is a severe and irreversible lung remodeling process that is possibly life-threatening. Therefore, rapid diagnosis and early, aggressive treatment are essential. However, the correct identification of high-risk candidates for PF in pediatric practice remains challenging. The evaluation of clinical parameters in conjunction with radiologic and histologic findings often fails to provide conclusive evidence in favor of a specific, clear diagnosis with predictable outcome. Expanding the diagnostic toolbox to include genetic markers and linking them to a clinically well-defined picture can help guide medical decision-making and was the overarching objective for this work.

During this study, we set out to analyze a clinical cohort with PF and additional multisystemic disease features. We investigated a genetically defined but underexplored multisystem disease (Hermansky-Pudlak syndrome type 2) and refined current chILD classification by identifying previously unknown genetic entities that can potentially lead to a fibrosing phenotype in childhood (*FARSA/FARSB*, *ZNFX1*). The diseases presented herein emerge from different molecular mechanisms, therefore expanding the biological understanding of PF.

3.1. Hermansky-Pudlak syndrome type 2 - an organelle dysfunction in alveolar epithelial type 2 cells linked to childhood pulmonary fibrosis

In the first chapter, we provide an in-depth clinical and genetic characterization of Hermansky-Pudlak syndrome type 2 (HPS-2). Hermansky-Pudlak syndrome (HPS), first reported in 1959, is a rare hereditary multisystem disorder that comprises a spectrum of different subtypes[43-45]. Clinically, it is characterized by the core features of oculocutaneous albinism and bleeding diathesis[44]. Various additional symptoms such as granulomatous colitis, immunodeficiency, or interstitial lung disease with PF might exist depending on the specific disease subtype[44].

Currently, 10 subtypes are described, which are caused by bi-allelic mutations in human genes HPS1–HPS10[46]. Recently, an additional gene (HPS11) has been proposed[47]. These genes encode different protein complex subunits necessary for lysosome-related organelle biosynthesis and the trafficking of vesicular cargo[48,49]. The specific symptoms observed in HPS such as hypopigmentation and extended episodes of bleeding can be explained by a defective synthesis of lysosome-related organelles such as pigment-storing melanosomes

in the skin and the eye or dense granules in platelets, secretory organelles involved in blood coagulation[44,49].

In the lung, alveolar epithelial cell type 2 (AEC2) cells produce lamellar bodies (LBs), lysosome-related organelles that transport surfactant into the alveolar lumen[50]. Pulmonary surfactant is a mixture of lipids and proteins that covers the alveolar space and plays a crucial role in lowering surface tension at the air-liquid interface, which stabilizes alveolar structure[51]. In addition, surfactant has important immunomodulatory functions[52].

The development of HPS-PF is particularly prevalent among HPS-1, HPS-2, and HPS-4, which affect children and young adults (HPS-2) or middle-aged adults (HPS-1, HPS-4)[48,53]. The fibrosis pattern in HPS shares features with IPF in radiologic and histologic appearance, most importantly the IPF-typical UIP pattern[48]. However, unlike IPF, HPS-PF is characterized by a foamy swelling of AEC2 cells and the formation of giant LBs, assumed to form by fusion of multiple smaller LBs[54]. In a HPS mouse model, these LBs accumulate across the lifespan of the animal due to a defect in LB secretion[55]. In both humans and mice, this particular pathology leads to increased lysosomal and endoplasmic reticulum (ER) stress, and increased AEC2 apoptosis[56].

Apoptosis of AEC2 cells is critical, since AEC2 cells, in addition to their role in surfactant metabolism, also serve as a stem cell reservoir in the adult lung[57]. They are capable of both self-renewal and differentiation into alveolar epithelial cell type 1 (AEC1) cells, responsible for gas exchange[57]. Therefore, AEC2 cells are essential for lung regeneration after tissue injury and the maintenance of alveolar epithelial integrity[57,58]. The importance of AEC2 health is corroborated by the findings that targeted injury of AEC2 cells causes spontaneous fibrosis in mice and that their senescence drives progressive PF[59,60]. Similarly, mouse models of relevant human mutations or deficiencies impacting AEC2 integrity develop fibrosis as well[61-63]. For example, telomere dysfunction, specifically induced in AEC2 cells, is sufficient to cause age-dependent lung remodeling and fibrosis in mice[62]. Moreover, AEC2 cell dysfunction is well documented in human genetic defects of surfactant production linked to fibrosis but also evident in sporadic IPF, suggesting a common pathomechanism[64,65]. Concordantly, HPS mouse mutant studies demonstrate increased AEC2 cell apoptosis that correlates with fibrotic susceptibility to chemical challenge with the profibrotic stimulant bleomycin[66].

In conclusion, a convincing body of evidence demonstrates a strong link between AEC2 integrity and the development of fibrosis[67]. It is therefore reasoned that PF, in its essence, represents the end stage attempt to repair a compromised alveolar epithelium that has exhausted its capacity for self-renewal or regeneration[58,67]. Indeed, alveolar epithelial cell integrity appears to exist on a spectrum that is determined by individual genetics and age. In a cohort of adult PF patients, genetic variants with stronger impact on AEC2 health promote fibrosis earlier

in life, e.g. surfactant genes (median age 45 years)[68]. In turn, variants of milder effect contribute to fibrosis later in life, e.g. telomere genes (median age 62 years)[68]. The different forms of HPS interstitial pneumonia manifesting in childhood or adulthood could be located accordingly on the same spectrum.

3.2. FARS1-related recessive disease - variants in aminoacyl-tRNA synthetase genes connected to childhood pulmonary fibrosis

In the second and third chapter, we link bi-allelic variants in *FARSA* and *FARSB* to a novel recessive multisystem disorder of the aminoacyl-tRNA synthetase (ARS) disease spectrum. ARSs fulfill an essential role in protein biosynthesis by charging a tRNA with its cognate amino acid, which constitutes their canonical aminoacylation function[69]. As fundamental links between the nucleic acid and the protein world, ARSs have originated very early in evolution and are universally distributed across cellular life[69-71]. In humans, two subsets of ARSs are distinguished based on their cytoplasmic or mitochondrial localization[72,73]. With rare exception, cytoplasmic and mitochondrial ARSs are encoded by separate genes, unique for each amino acid[72,73]. They are abbreviated with their respective single letter amino acid code followed by "ARS" with the addition of "1" or "2" for cytosolic or mitochondrial forms, respectively[72]. *FARSA* and *FARSB* encode different subunits (alpha, beta) of the heterotetrameric cytosolic phenylalanyl-tRNA synthetase (FARS1)[74].

Throughout evolution, ARSs have gained functions beyond their translational activity, referred to as their non-canonical functions[75]. This correlates with the progressive acquisition of novel domains and motifs unrelated to aminoacylation and the introduction of alternative functions for ancient domains[75,76]. In addition, potential ARS functions beyond translation are supported by the large number of splice variants disrupting the catalytic domain[77]. Expanded ARS functions play a key role in a variety of signaling pathways including angiogenesis, inflammation and immune response, tumorigenesis, metabolic regulation, and proliferation[78].

The genetics of ARSs is complex with a multitude of distinct disease phenotypes reported to date[79]. Among these, three main groups have emerged as a cause for rare inherited disease, including recessive mitochondrial disorders, recessive multisystem disorders, and dominant axonal neuropathies of the Charcot-Marie-Tooth (CMT) type[72,79,80]. While CMT pathology is limited to the peripheral nervous system, mitochondrial ARS mutations clinically affect the central nervous system (CNS) and/or to a lesser degree additional systems that are partially dependent on CNS function[81]. Cytoplasmic ARS mutations typically display a broader phenotypical spectrum affecting multiple organs, mostly with a neurological component[79].

To what extent the defects in aminoacylation, seen in many recessive ARS disorders, translate into the various organ-specific phenotypes has been controversially discussed[79,82]. It has been hypothesized that such effects might be based on unmet tissue-specific translational demands in selected amino acids[82]. For example, CARS1 variants cause brittle hair and nails, tissues with proteins particularly enriched in cysteine[82,83]. On the other hand, certain disease phenotypes could be related to specific subfunctions of ARSs. For example, LARS1 variants cause infantile liver syndrome[84,85]. LARS1 is a regulator of mTORC1 signaling, a pathway that induces autophagy and is associated with liver dysfunction[82,86-88]. In agreement with both theories, supplementation with specific amino acid improves the pathology in some organs, but not all[89]. Interestingly, variants in the editing domain of AARS1 cause cardioproteinopathy and fibrosis, based on the accumulation of misfolded proteins due to translational infidelity of the ARS enzyme, adding yet another potential mechanism to tissue-specific pathologies[90].

To date, variants in MARS1, IARS1, LARS1, and FARS1 have been associated with the development of alveolar proteinosis, a rare, specific subtype of ILD[73,91-93]. Alveolar proteinosis is characterized by the accumulation of lipoproteinaceous material derived from surfactant caused by altered surfactant production, removal or both[94]. In the cases of MARS1 and IARS1, variant expression in yeast shows reduced amino acid incorporation for methionine (MARS1) and/or impaired growth (MARS1, IARS1)[91,95]. For MARS1, this deficiency can be corrected by amino acid supplementation[91]. Accordingly, the pulmonary symptoms in two MARS1 patients improved with amino acid supplementation and protein-rich diets[96]. Likewise, beneficial effects of isoleucine supplementation for two IARS1 patients have been reported[89,95].

On the other hand, FARS1 cases typically develop cholesterol pneumonitis, which is accompanied by alveolar proteinosis in some[73]. This condition is characterized by the accumulation of intra-alveolar lipid and lipid-laden macrophages and has been documented in association with bronchial obstruction in lung cancer patients, but also for different other conditions including genetic disease[97]. With the exception of one case, compromised aminoacylation could be shown for all FARS1 patients studied to date[73,89,93]. However, whether impaired aminoacylation leads to global insufficiency in protein synthesis remains to be determined. On the clinical level, amino acid supplementation in two FARS1 patients with demonstrated aminoacylation defects did not improve their respiratory condition, even though advanced fibrotic remodeling in one patient might have prevented such effects[73,89]. These data, in combination with primary cell studies that suggest sufficient protein synthesis and cell growth in spite of impaired aminoacylation, indicate that the FARS1-related recessive phenotype might possibly be linked to a function beyond protein biosynthesis[93]. Besides uncovering tissue-specific effects of impaired aminoacylation and their consequences for protein synthesis, defining potential non-canonical ARS functions and their relationship to disease will therefore be a major focus of future studies in the field.

Novel insights into the basic biology of ARSs will help advance understanding of rare inherited disease prevalent in childhood. However, they will also benefit adult medicine. Specifically, antibodies against a range of ARS, including FARS1, are present in a rheumatological disease called "anti-synthetase syndrome"[98-100]. This condition is characterized by symptoms of inflammatory myopathy, arthritis or arthralgia, fever, specific cutaneous signs, and ILD[101]. The prognosis is largely determined by the pulmonary pathology, which remains, however, poorly understood to date[102,103].

3.3. ZNFX1 deficiency - a defective viral sensor associated with immune dysregulation and childhood pulmonary fibrosis

In the fourth chapter, we describe a novel inborn error of immunity with features of type 1 interferonopathy due to loss of function in ZNFX1. Type 1 interferonopathies are multisystem disorders of the innate immune system that are caused by dysregulation of type 1 interferon (IFN) signaling, an important signaling pathway in immune cell activation and protection against viruses[104,105]. Despite being phenotypically heterogenous, this disease group is clinically most represented by the "IFN signature" symptoms of skin vasculopathies with chilblains, livedo reticularis and panniculitis, brain involvement, and ILD[105].

Monogenic type 1 interferonopathies are caused by loss of function in gene products involved in viral and endogenous nucleic acid sensing and metabolism or gain of function in positive IFN signaling regulators, leading to constitute activation of the IFN pathway[105]. Alternatively, dysregulation of IFN signaling can occur through loss of function of negative IFN signaling regulators or proteasomal dysfunction, leading to increased IFN signaling through a yet unknown mechanism[105]. Among the group of interferonopathies, some develop ILD with PF, e.g. stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI) or coatomer protein subunit alpha (COPA) syndrome[106]. ZNFX1 is a recently discovered viral sensor that recognizes viral nucleic acids and regulates the type 1 IFN response[107]. Upon viral infection, ZNFX1 upregulates IFN signaling, therefore promoting viral clearance[107,108]. On the other hand, ZNFX1 decreases the half-life of interferon-stimulated genes, therefore helping to resolve inflammation[108]. In line with both functions, ZNFX1 deficiency creates a phenotype of increased susceptibility to viral infection in some patients and chronic multisystem inflammation in others[108].

The related type 1 interferonopathy SAVI is characterized by early-onset systemic inflammation, (fibrosing) ILD, and cutaneous vasculopathy due to autosomal dominant variants in the *STING1* gene, which encodes STING, a central regulator of IFN signaling[109]. SAVI mouse models present phenotypically similar to humans with chronic inflammation, paw

swelling, necrosis of tail and ear, skin lesions, and pulmonary inflammation/fibrosis[110-112]. As in their human counterparts, they exhibit upregulation of IFN or IFN-stimulated genes and T cell cytopenia[110-112]. Most importantly, mouse studies demonstrate that lung disease develops independently of type 1 IFN signaling and relies on T cells[113]. The increased T cell death is promoted by a disruption of calcium homeostasis, subsequently increased ER stress, and the unfolded protein response. Upon restoration of $CD8^+$ T cells, STING-associated lung disease drastically improves[114].

In a similar vein, COPA syndrome in human is characterized by (fibrosing) ILD, joint and kidney disease, and a prominent IFN signature, features that are broadly overlapping with SAVI[28,60]. Interestingly, COPA encodes a coatomer protein involved in vesicular trafficking of STING and STING-dependent induction of type 1 IFN[115]. Furthermore, a COPA missense variant mimicking the human condition in mice leads to a defect in thymic selection of T cells, leading to an escape of autoreactive T cells and a decreased number of regulatory T cells in the periphery[116].

These examples show that both innate and adaptive immune responses can be affected in type 1 interferonopathies. Emerging insights from this relatively young but evolving field will continue to inform related areas of phenotypically similar but genetically complex rheumatologic diseases that can result in PF, e.g. systemic lupus erythematosus or (juvenile) dermatomyositis[117]. In fact, Mendelian type 1 interferonopathies, as described here, are considered monogenic forms of lupus or lupus-like disease[118]. On the other hand, diseases such as juvenile dermatomyositis are considered acquired forms of interferonopathy[119].

3.4. Children at high risk for pulmonary fibrosis - early diagnosis is key

The recognition of distinct well-known and genetically defined entities of disease is essential, as it allows not only the rapid identification necessary for patients at high risk for fibrosis, but it also offers mechanistic insights, recommendations for disease management, and options for personalized treatment. Rare disease patients can spend years on time-consuming diagnostic odysseys, which places a significant burden on their families and local health care resources[120]. In addition, late diagnosis limits possibilities for medical intervention within a critical time window and timely enrolment in clinical trials.

Multisystemic diseases in particular need interdisciplinary care and organ systems expected to be at risk for disease require thorough and regular monitoring. A deeper understanding of disease origin can have immediate implications for clinical practice. For example, awareness of an immunological dysfunction such as ZNFX1 deficiency translates into specific considerations regarding virus exposure, vaccine administration, and medical

treatment. Genetic analysis can now guide these differential diagnoses and reduce the risk of invasive procedures such as biopsies, that are especially critical to the immunocompromised. In addition, genetic markers bring the benefit of being, in many cases, more exact and reliable diagnostic measures compared to clinical parameters and radiologic or histologic patterns, that can be heterogenous and occasionally misleading depending on the clinical state of the patient and the diagnostic experience of the evaluating physician.

In conclusion, international collaborative rare disease registries such as chILD-EU are key to expediting the diagnostic process by providing access to consultation with chILD experts and tailored diagnostic tools. Cases with similar clinical phenotypes can be identified, collated to cohorts, and explored in detail on a research basis. The resulting data will help raise awareness of existing rare disease diagnoses among primary health care providers, genetic counselors, and affected families. Most importantly, the output serves to educate in management of previously little-known or unknown disease entities once a diagnosis is made.

3.5. From sequence variant to genetic disorder - linking genetic information to human disease

High-throughput sequencing methods, as employed in this study, generate large amounts of genetic data. However, despite careful evaluation, distinguishing potentially disease-causing genetic variants from a broad population control background in order to generate meaningful clinical information comes with its challenges[121]. Even though statistical and informatic analyses can help to predict and interpret the significance and effect of variants, their limitations must be taken into account when interpreting results from a biological perspective[121]. For example, conservation scores, as frequently used for the evaluation of deleteriousness in genetic variation, might not have strong predictive power for genes that have undergone rapid evolution[121]. Likewise, they might be misleading in the case of pathogenic amino acid substitutions in proteins that form compensatory substitutions in different species[121,122]. Moreover, selection of candidate genes for disease may be complicated in consanguineous families with a high number of homozygous variants, that might be both disease-related and -unrelated, as well as in patients from ethnic backgrounds currently underrepresented in genetic control databases[123]. In addition, strongly heterogeneous phenotypes can often be perceived as differing entities, making phenotypic attribution to distinct genetic variants more likely. Particularly difficult to interpret are variants of gene products with unknown function or little biological context, as well as variants in ubiquitously expressed proteins with general or multiple functions that are not obviously connected to certain cell type- or tissue-specific functions. Given the unique genetic background of each patient and the rarity of the conditions,

but also the pulmonary focus of this study, it is important to recognize that the clinical picture of the described phenotypes can change as more data is gathered over time.

In order to support a causal relationship between an identified genetic variant and a certain phenotype, further experimental studies investigating the effect of the mutant gene product, as well as mechanistic studies in relevant cell models, possibly supplemented by biochemical studies, will be needed[123]. The establishment of animal models that recapitulate the human phenotype can be helpful in studying cellular processes on the organismic level and during specific phases of development[123]. The gradual improvements of knock-down and transfection techniques for primary cells and cell lines, the recent advent of genome-editing technologies, and the lately available stem cell-based systems, e.g. lung organoids, represent new ways that can leverage molecular understanding of the precise relationship between distinct genotypes and disease[123-125]. In combination with the rise of "multi-omics" technologies, these tools will certainly help to further disentangle the roles of genetic, molecular, and environmental factors at the basis of complex pathological lung remodeling processes such as fibrosis[126]. In doing so, this and other studies might contribute to solving central questions regarding the nature of fibrosis way beyond the pediatric field. Specifically, they may shed light on how different forms of fibrosis are related and how childhood and adult ILD might represent variable expressions of the same underlying gene pathogenic mechanism reaching from childhood into adulthood[68,127].

3.6. Treatment of pulmonary fibrosis in childhood - current practices and future perspectives

Current treatment for chILD typically involves immunomodulatory drugs that are administered to suppress inflammation and prevent potential further progression into fibrosis[33]. Supportive care includes supplemental oxygen, routine vaccination, non-exposure to pollutants or known irritants, and infection treatment[33,128]. Severe cases of PF are considered for lung transplant[129]. In adults, approved drugs for (I)PF treatment comprise the molecules pirfenidone and nintedanib, which inhibit central pathways implicated in fibrosis and have proven effective in preventing decline of lung function in phase 3 clinical trials[130-135]. A clinical trial of nintedanib in children and adolescents evaluating dosing and safety is presently ongoing[136].

In parallel, recent developments in molecular medicine have paved the way for more tailored treatment approaches for monogenic diseases. Significant progress has been made in the field of genetic engineering giving rise to unprecedented possibilities for whole genome manipulation including transcription activator-like effector nucleases (TALENS), zinc finger nucleases (ZFN), and clustered regularly interspaced short palindromic repeats (CRISPR)/

CRISPR-associated (Cas) proteins[124]. In conjunction with stem cell techniques and transplantation, these techniques hold great promise in revolutionizing the field of gene therapy[124,137].

The drugs emerging from these new technologies are based on precise genome sequence correction and constitute the latest addition to a growing collection of gene therapy products on the market[138,139]. The feasibility of viral transgene delivery had been the focus of previous works in the field and lead to the approval of a number of drugs for treatment of hereditary conditions[138,139]. Of interest, lentiviral-mediated gene transfer of the *HPS1* gene, which is deficient in Hermansky-Pudlak type 1, restores HPS1 expression and function in patient melanocytes providing a first proof of principle for the *in vitro* correction of a gene implicated in PF[140].

For all gene therapy approaches, target cell types and potential delivery systems must be carefully evaluated[137]. Even though the lung is a well-accessible organ, pulmonary transgene delivery has traditionally been hampered by various physical and immunological barriers[141-143]. These include, among others, the continuous removal of foreign particles by mucociliary clearance mechanisms and alveolar macrophages and a natural preexisting immunity to respiratory viruses with lung tropism that are frequently used as transgenic vectors[141-143]. In addition, progenitor cells of the lungs, needed for long-time transgene expression, can be difficult to access or target[143-145].

In contrast, the immune system is naturally more amenable to genetic manipulation and correction of genetic defects has been shown for several primary immunodeficiencies[146]. As monogenic primary immune system disorders, type I interferonopathies might in principle be candidates for gene therapy. However, more insights into the pathomechanistic processes within this disease group will be needed to understand the effects of genetic variants and map potential interactions between different cell types and tissues in order to begin developing tailored treatment options[147].

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5. Appendix

5.1 Abbreviations

α-SMA	α-smooth muscle actin
AARS	alanyl-tRNA synthetase
aaRS	aminoacyl-tRNA synthetase
ABCA3	ATP-binding cassette subfamily A member 3
ADAR	adenosine deaminase, RNA specific
ADP	adenosine diphosphate
ADV	adenovirus
AEC1	alveolar epithelial cell type 1
AEC2	alveolar epithelial cell type 2
aHUS	atypical hemolytic uremic syndrome
AIMP	aminoacyl-tRNA synthetase-interacting multifunctional protein
ALAT	alanine aminotransferase
AMP	adenosine monophosphate
AP3B1	adaptor-related protein complex 3 subunit beta-1
AP3B3A	AP-3 complex beta-3A subunit
ARDS	acute respiratory distress syndrome
ARS	aminoacyl-tRNA synthetase
ASAT	aspartate aminotransferase
ASDII	atrial septal defect II
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
ATP11A	ATPase, phospholipid-transporting 11A
AZI2	5-acacytidine-induced protein 2
BAL	bronchoalveolar lavage
BCG	bacillus Calmette-Guérin
BIRC4	baculoviral IAP repeat-containing protein 4
BM	bone marrow
BMI	body mass index
CARS	cysteinyl-tRNA synthetase
Cas	CRISPR-associated
CCL	C-C motif chemokine ligand

CDC42	cell division control protein 42
CDG	congenital disorder of glycosylation
CDKN1A	cyclin-dependent kinase inhibitor 1A
cGAS	cyclic GMP-AMP synthase
chILD	childhood interstitial lung disease
chILD-EU	European management platform for childhood interstitial lung diseases
CMTD	Charcot-Marie-Tooth disease
CMV	cytomegalovirus
CNS	central nervous system
cNSIP	cellular nonspecific interstitial pneumonitis
COPA	coatomer protein subunit alpha
CP	cyclophosphamide
CRISPR	clustered regularly interspaced short palindromic repeats
CSA	cyclosporine A
CSF	cerebrospinal fluid
CT	computer tomography
CTD	connective tissue disease
CTRL	control
CV	central veins
CXCL10	C-X-C motif chemokine 10
DHX58	DEXH-box helicase 58
DIP	desquamative interstitial pneumonitis
DKC1	dyskerin pseudouridine synthase 1
DLCO	diffusing capacity of the lung for carbon monoxide
DMEM	Dulbecco's Modified Eagle Medium
Do	donor
DPP9	dipeptidyl peptidase 9
DRB	5,6-dichlorobenzimidazole 1-β-D ribofuranoside
dsDNA	double-stranded DNA
DSP	desmoplakin
EBV	Epstein-Barr virus
EBV-LCL	Epstein-Barr virus-immortalized lymphoblastoid cell line
ECM	extracellular matrix
ECMO	extracorporeal membrane oxygenation
EEG	electroencephalogram
EIF2AK2	eukaryotic translation initiation factor 2 alpha kinase 2
EMH	extramedullary hematopoiesis

ER	endoplasmic reticulum
EvG	Elastica van Gieson
f	female
FACS	fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FARS	phenylalanyl-tRNA synthetase
FARS1	cytosolic phenylalanyl-tRNA synthetase
FARSA	phenylalanyl-tRNA synthetase subunit alpha
FARSB	phenylalanyl-tRNA synthetase subunit beta
FBS	fetal bovine serum
FEF25-75	forced expiratory flow between 25%-75% of the FVC
FEV1	forced expiratory volume of first second
Fib	fibrinogen
FLAIR	fluid-attenuated inversion recovery
FU	follow-up
FVC	forced vital capacity
G-CSF	granulocyte colony-stimulating factor
GAPDH	glyceraldehyde-3- phosphatase dehydrogenase
GARS	glycyl-tRNA synthetase
GGO	ground-glass opacities
GGT	gamma glutamyl-transferase
GMP	guanosine monophosphate
GRP78	78-kDa glucose-regulated protein
GTP	guanosine triphosphate
HAVCR2	hepatitis A virus cellular receptor 2
HE	hematoxilin-eosin
HEM1	hematopoietic protein 1
HHV6	human herpes virus type 6
HLH	hemophagocytic lymphohistiocytosis
HP	hypersensitivity pneumonitis
HPRT	hypoxanthine-guanine-phosphoribosyl-transferase
HPS-1-10	Hermansky-Pudlak syndrome type 1-10
HRCT	high-resolution computed tomography
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
IARS	isoleucyl-tRNA synthetase
ID	identifier

IFIH1	interferon-induced with helicase C domain protein 1
IFIT1/2	interferon-induced protein with tetratricopeptide repeats 1/2
IFN	interferon
IGRA	interferon gamma release assay
IKK	inhibitor of nuclear factor kappa-B kinase
IL1RN	interleukin 1 receptor antagonist
IL8	interleukin 8
ILD	interstitial lung disease
INR	international normalized ratio
iNSIP	idiopathic nonspecific interstitial pneumonia
IPAF	interstitial pneumonia with autoimmune features
IPF	idiopathic pulmonary fibrosis
IRF1/7	interferon regulatory factor 1/7
IRF7	interferon regulatory factor 7
ISG	interferon-stimulated gene
IVIG	intravenous immunoglobulins
JAK1/2	Janus kinase 1/2
kDA	kilodalton
KIF15	kinesin family member 15
LB	lamellar body
LDH	lactate dehydrogenase
LLL	left lower lobe
LUL	left upper lobe
LYST	lysosomal trafficking regulator
m	male
MAD1LI	mitotic arrest-deficient 1-like 1
MAF	minor allele frequency
MAP1LC3B	microtubule-associated protein 1 light chain 3B
MARS	methionyl-tRNA synthetase
MAVS	mitochondrial antiviral-signaling protein
MDA5	melanoma differentiation-associated protein 5
MFI	median fluorescence intensity
Min	minute
ML	middle lobe
MMF	mycophenolate mofetil
MMP	matrix metalloproteinase
MMR	mumps, measles, rubella

Mo	month
MOF	multiorgan failure
MORC3	MORC family CW-type zinc finger 3
MOV10	Mov10 RISC complex RNA helicase
MPGN	membranoproliferative glomerulonephritis
MRI	magnetic resonance imaging
MSC	multi-synthetase complex
mTOR	mammalian target of rapamycin
MTX	methotrexate
MUC2	mucin 2
MUC5B	mucin 5B
MX1	MX dynamin-like GTPase 1
MYD88	MYD88 innate immune signal transduction adaptor
NA	not available
Nd	not done
NFκB	nuclear factor kappa-B
NFX-1	nuclear transcription factor, X-box binding 1
NGS	next generation sequencing
Nk	not known
NK cells	natural killer cells
NLRC4	NLR family CARD domain-containing 4
OAS1/2	oligoadenylate synthetase 1/2
OBFC1	oligonucleotide/oligosaccharide-binding fold-containing protein 1
OPV	oral poliovirus vaccine
PALF	pediatric acute liver failure
PAMP	pathogen-associated molecular patterns
PARN	poly(A)-specific ribonuclease
PARP12	poly (ADP-ribose) polymerase family member 12
PAS	periodic acid-Schiff
PBMC	peripheral blood mononuclear cells
PEG	percutaneous endoscopic gastrostomy
PF	pulmonary fibrosis
Phe	phenylalanine
PML	promyelocytic leukemia protein
PRF1	perforin 1
Pro-SP-B	pro-surfactant protein B
Pro-SP-C	pro-surfactant protein C

PTT	partial thromboplastin time
PVDF	polyvinylidene fluoride
RAB27A	Rab27A, member RAS oncogene family
RC3H1	ring finger and CCCH-type domains 1
RIG-1	retinoic acid-inducible gene 1
RIPA	radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RLL	right left lobe
RNF114	ring finger protein 114
RPL9	ribosomal protein L9
RPS11	ribosomal protein S11
RSAD2	radical S-adenosyl methionine domain-containing 2
RSV	respiratory syncytial virus
RTEL1	regulator of telomere elongation helicase 1
RTI	respiratory tract infection
RUL	right upper lobe
SAP	SLAM-associated protein
SARS-CoV2	severe acute respiratory syndrome coronavirus type 2
SAT1	spermidine/spermine N1-acetyltransferase 1
SAVI	STING-associated vasculopathy with onset in infancy
SFTPA1	surfactant protein A1
SFTPA2	surfactant protein A2
SFTPC	surfactant protein C
SH2D1A	SH2 domain-containing protein 1A
SHISA5	Shisa family member 5
SJV	splice junction variant
SLAM	signaling lymphocyte activation molecule
SP	surfactant protein
SQSTM1/P62	sequestosome 1/ ubiquitin-binding protein p62
ssRNA	single-stranded RNA
STAT1/2	signal transducer and activator of transcription 1/2
STING	stimulator of interferon genes
STX11	syntaxin 11
STXBP2	syntaxin-binding protein 2
TALENS	transcription activator-like effector nucleases
TERC	telomerase RNA component
TERF1	telomeric repeat-binding factor 1

TERT	telomerase reverse transcriptase
TG	triglycerides
TGFB1	transforming growth factor beta 1
TINF2	TERF1-interacting nuclear factor 2
TLC	total lung capacity
TLR3	toll-like receptor 3
TMA	thrombotic microangiopathy
TOLLIP	toll-interacting protein
TP53	tumor protein 53
TRIF	toll-like receptor adaptor molecule 1
TRIM25	tripartite motif-containing protein 25
uILD	unclassifiable interstitial lung disease
UIP	usual interstitial pneumonia
UNC13D	Unc-13 homolog D
UTR	untranslated region
VP16	Herpes simplex virus protein Vmw65
VSV	vesicular stomatitis virus
VZV	varicella-zoster virus
WBC	white blood cell count
WES	whole exome sequencing
XIAP	X-linked inhibitor of apoptosis protein
Y	years
ZC3HAV1	zinc finger CCCH-type-containing, antiviral 1
ZFN	zinc finger nucleases
ZNFX1	zinc finger NFX1-type containing protein 1

5.2 Declaration of contribution

Contributions are listed according to CRediT (Contributor Role Taxonomy)[148].

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Xu, Zhiwen: investigation and formal analysis (protein expression analysis, protein synthesis assay, involved in prediction of mutational impact on protein structure), writing (original draft, revisions)

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Lo, Wing-Sze: investigation, formal analysis, validation (contributions to results in Fig.4 and Fig.5A, including first validation of *FARSB* splice site mutation c.848+1 G>A in the patient family, prediction of splicing consequence of this mutation [Fig.4A], identification and detection of the *FARSB* splice variants in patient and family members, validation of the splicing consequence prediction [Fig.4B], all quantification of gene expression in the study, such as transcript levels in Fig.4C and Fig.5A), resources (cultivation of fibroblast cells for *in vitro* experiments)

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Schuch, Luise A.: investigation and formal analysis (clinical phenotyping, WES analyses), resources (organization, generation, and preparation of patient/family biomaterials for analysis, cultivation of patient-derived primary cells), manuscript review and editing, supervision and project administration support (collaboration management, communication, oversight, and coordination of internal and external research activities)

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I confirm that the statements of contribution to authorship are correct.

…….

(M. Griese)

5.3 Statutory declaration and statement

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt ist.

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

München, den 03.02.2022 …….....

(Luise A. Schuch)

5.4 Acknowledgements

The work presented here has been an absolute team effort and would not have been possible without the hard work of a number of people, many of whom have been involved in essential preparations leading up to this research.

I would like to acknowledge Prof. Dr. Matthias Griese for his work in the establishment and continuous maintenance and improvement of the chILD-EU registry over many years. He and countless other collaborators provided me with a goldmine of unique clinical cases to study, which was a fantastic learning experience. Thank you, Matthias, for trusting me with the challenge to explore a new research field and giving me the freedom and resources to find my own projects and see them evolve.

I would like to thank Prof. Dr. Christof Osman and Prof. Dr. Bertram Müller-Myhsok for being part of my advisory committee and accompanying my research steps and ideas with helpful discussions and input. In addition, I would like to thank all examiners of this thesis for their immediate interest in the topic of chILD.

Thank you to all the project collaborators who joined forces and shared expertise to advance rare disease research as well as to everyone providing infrastructure to connect research groups around the globe. A heartfelt thank you goes to all the clinicians, nurses, and administrative staff for organizing precious rare patient samples. I would also like to gratefully acknowledge my colleagues at the Klein group at the Dr. von Hauner Children's Hospital for providing help in genetic analysis and training in essential cell culture techniques.

I highly appreciate all the support I have received from the members of the Griese group. Thank you to my physician colleagues, who, despite their busy schedules, always took some time to answer my medical questions and teach me about pediatrics. Thank you to all my talented lab colleagues who helped me, worked alongside me, and became my friends and companions throughout the years. A special thank you goes to W. Wesselak and A. Schams for their kind technical and logistical support.

Most importantly, I would like to thank my family and friends for their patience and love. Thank you for keeping me grounded and always reminding me that the world is a vast and colorful place that has many worthwhile things to offer that do not necessarily involve research. To André, I am deeply grateful for your generosity, understanding, and steady company.

Lastly, to the rare disease community. Thank you for all your kind donations and support for this research. Many of you have been through the unimaginable. Yet your courage and resilience are extraordinary. This thesis really is for you.