TOWARDS NEW GUIDELINES FOR DIAGNOSIS AND TREATMENT OF PRIMARY ANTIBODY DEFICIENCY:

Using translational studies to uncover new diagnostic and therapeutic modalities

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Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Layout and design: Jules Verkade, persoonlijkproefschrift.nl
Cover design: Koyo Do

DOI: https://doi.org/10.33540/2010
ISBN: 978-94-6483-448-2

Sponsorinfo:

Sponsoring: This thesis was sponsored by Infection & Immunity Utrecht.

Towards new guidelines for diagnosis and treatment of primary antibody deficiency:

Using translational studies to uncover new diagnostic and therapeutic modalities

Richting nieuwe richtlijnen voor het diagnosticeren en behandelen van primaire antistof defecten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 15 november 2023 des middags te 4.15 uur

door

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geboren op 17 januari 1990
te Utrecht

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TABLE OF CONTENTS

CHAPTER 1

General Introduction

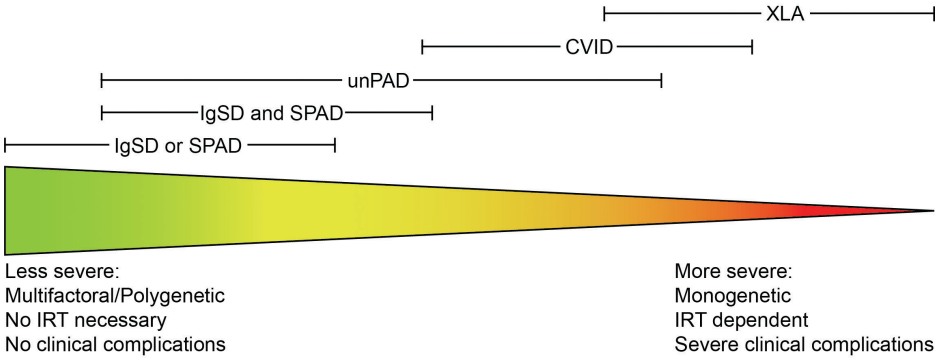
PRIMARY ANTIBODY DEFICIENCY

Symptomatic primary antibody deficiencies (PAD) are the most common symptomatic form of primary immunodeficiency. [1,2] They are estimated to occur in 1:2.000 – 1:10.000 individuals worldwide and usually present before the age of 30 years.[1,2] PAD is a heterogenous group of diseases characterized by reduced immunoglobulin production, that can be further classified based on the types of immunoglobulins that are missing and the severity of the immunoglobulin deficiency. [3–5]

In specific polysaccharide antibody deficiency (SPAD) and immunoglobulin G (IgG) subclass deficiency (IgSD) patients, only specific immunoglobulins or IgG subclasses are lacking,[5] whereas in common variable immunodeficiency (CVID) and (x-linked) agammaglobulinemia (XLA), B cells are not able to adequately produce entire classes of immunoglobulins or are incapable of producing any immunoglobulins.[5] Thus, the pathophysiology of PAD is related to B cell dysfunction.[3,4]

The classification of PAD is related to the severity of B cell dysfunction (Figure 1).[5] Here, B cell dysfunction leading to isolated IgSD or SPAD is deemed the least severe form of PAD followed by dysfunction leading to combined IgSD and SPAD or unclassifiable hypogammaglobulinemia (unPAD), whereas CVID, agammaglobulinemia and XLA are deemed more severe.[5,6] More severe types of PAD are often associated with earlier age of onset, monogenetic disease, and severe clinical complications such as infectious sequelae and non-infectious complications, and often requires more intensive infectious preventive measures and immunosuppressive therapy.[3,6,7]

Figure 1: *Schematic classification of PAD, the associated severity of B cell dysfunction and associated disease severity*



Infectious complications in PAD

Recurrent infections are frequently the presenting symptom in patients with PAD.[3,8] Normally, antibodies play an important role in the protection of mucosal surfaces.[3] Therefore, patients with PAD frequently suffer upper and lower respiratory tract infections typically caused by polysaccharide encapsulated bacteria like *Streptococcus pneumoniae* and *Haemophilus influenzae*.[9,10] Moreover, chronic diarrhea due to frequent gastro-intestinal infections caused by *Giardia lamblia*, *Salmonella enterica*, *Campylobacter jejuni*, and rotavirus have been described.[9,10] However, infections are not limited to mucosal tissues, and severe invasive infections such as meningitis and osteomyelitis have also been reported to occur more frequently in PAD patients.[9,10] Infection prevention measures like vaccination, prophylactic antibiotics and immunoglobulin replacement therapy (IRT) have been implemented successfully to combat infections in PAD and have greatly reduced the incidence of infectious complications among PAD patients.[11–13] Currently, IRT is the corner stone for infection prevention in CVID and XLA patients.[13] However, superior efficacy of IRT has not yet been proven for IgSD and SPAD and it is currently not known whether IRT or prophylactic antibiotics should be used as first-line infection prevention measure in these diseases.[14,15]

Despite the successful implementation of infection prevention measures, infectious sequelae still occur in PAD.[4] Bronchiectasis is the most important infectious sequela in PAD and is typically caused by recurrent lower respiratory tract infections and the subsequent structural damage of lung tissue.[16] A recent meta-analysis on bronchiectasis in CVID has found that bronchiectasis was reported on average in 34% of the patients in the included studies.[17] Bronchiectasis can cause chronic cough and dyspnea on exertion and may lead to an increase in lower respiratory tract infections and a decrease in pulmonary function.[18] Furthermore, bronchiectasis can result in more hospitalizations, decreased quality of life and end-stage pulmonary failure, and may eventually lead to early death.[19,20] Therefore, frequent pulmonary screening with computed tomography (CT) has been recommended for all PAD patients.[21] However, it is currently not known which PAD patients are at risk for bronchiectasis.

Other infectious sequelae of PAD are sensory hearing loss and malnutrition.[22,23] Sensory hearing loss in PAD is caused by recurrent acute otitis media.[22] One previous study has reported that sensory hearing loss was reported among 38% of CVID and XLA patients in a cohort of children and young adults.[22] Malnutrition in PAD can be caused by recurrent gastrointestinal infections. One study found

that malnutrition occurred in 16.3% of PAD patients in a cohort of children with primary immunodeficiency.[23] However, because of successful implementation of infection prevention measures, morbidity caused by infectious sequelae has been reduced in PAD.[24]

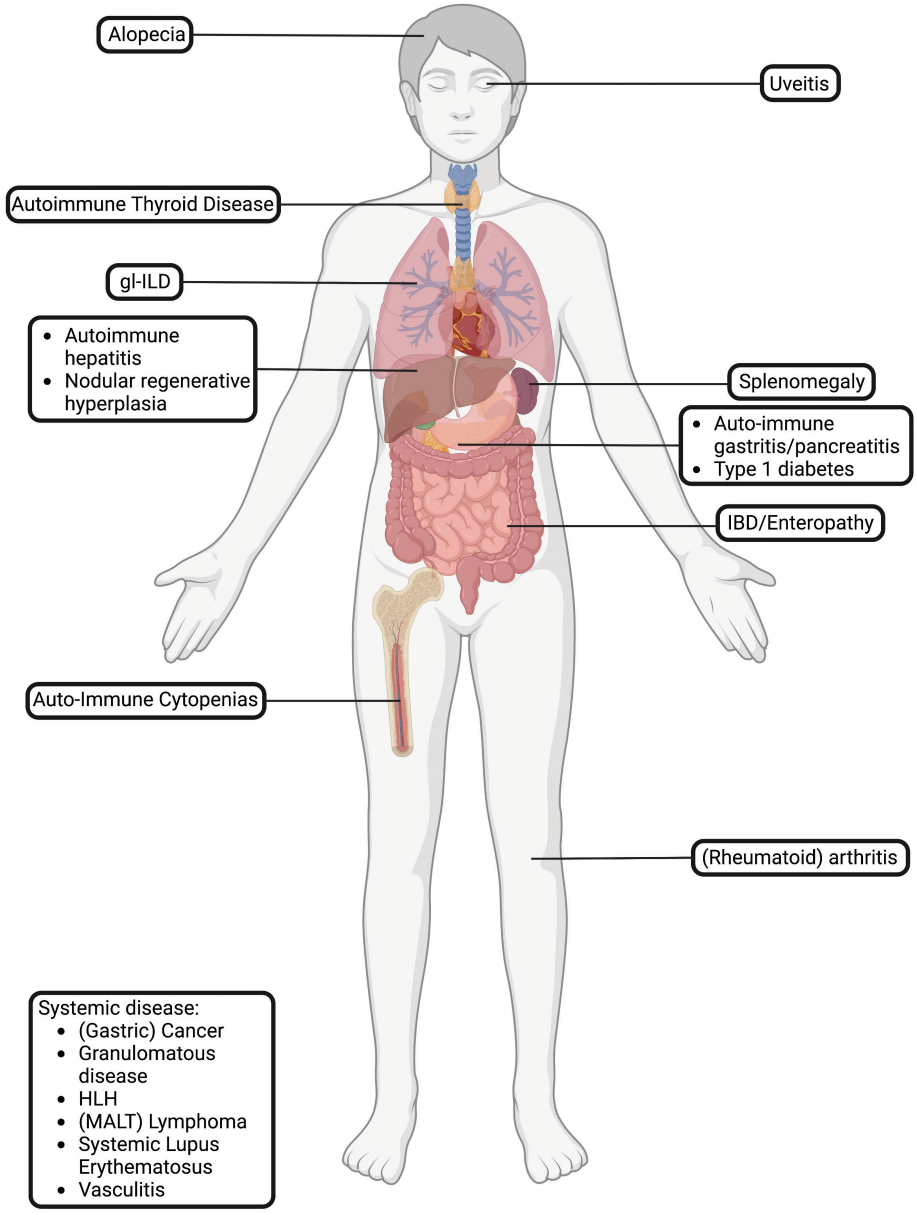
Non-infectious complications in PAD

Currently, non-infectious complications are the most important cause of morbidity and mortality among CVID patients and occur in 30% - 60% of patients.[7,25,26] These complications can affect a multitude of organ systems (Figure 2), and include lymphoproliferation, malignancies, and auto-immune and inflammatory manifestations, such as enteropathy, granulomatous or lymphocytic interstitial lung disease (GLILD) and secondary hemophagocytic lymphohistiocytosis (HLH).[26–28]

Enteropathy is a term that describes any gastrointestinal disease (GI) in CVID where an infectious cause has been excluded.[29] Enteropathy is associated with inflammation that can be both acute, inflammatory bowel disease-like (IBD-like) and chronic in nature and occurs in 20 - 30% of CVID patients.[29] It can cause diarrhea, bloating, reflux and pain and can lead to protein-loss and malnutrition.[29] Enteropathy often presents with lymphocytic colitis hallmarked by unspecific inflammation and infiltrates enriched in T cells and diminished in plasma cells.[29] Specific treatment for CVID related enteropathy has scarcely been described and is often ineffective.[29]

Granulomatous lymphocytic interstitial lung disease (GLILD) occurs in approximately 10-20% of CVID patients and can be asymptomatic, but can also cause non-specific symptoms such as cough and dyspnea on exertion.[20,30] Nodules, consolidations and ground glass abnormalities in the lower regions of the lung on high-resolution CT are highly suggestive of GLILD.[20,30] The pulmonary infiltrates have been shown to consist of B and T cells.[20,30] A variety of immunosuppressive regimens have been described that can potentially be used to treat GLILD.[31] However, treatment guidelines for GLILD as well as a systematic review of the evidence for these regimens is lacking.[20]

Figure 2: *Non-infectious complications of PAD (created with Biorender).*



Secondary HLH is a life-threatening immune dysregulation syndrome that can occur as a rare complication of CVID.[27,32] HLH is characterized by uncontrolled immune activation leading to multiple organ failure and death. In CVID, it can be triggered by severe infections caused by Epstein-Barr virus, cytomegalovirus,

influenza virus and *Staphylococcus spp.* as well as non-infectious complications like lymphoma, juvenile idiopathic arthritis (JIA) and systemic lupus erythematosus.[32] HLH is treated by treating the underlying trigger, combined with high dose immunosuppressive therapy.[32] Because of its severe nature, early treatment of HLH is imperative.[32] HLH is diagnosed when at least 5/8 of HLH-2004 criteria are present.[32] However, most of these criteria are non-specific and some criteria can be time consuming to acquire.[33,34] These two factors can contribute to diagnostic delay and can delay initiation of treatment.

Management of non-infectious complications in PAD

Currently, specific guidelines for the management of non-infectious complications in PAD are lacking.[26] Since PAD is a rare disease, it is difficult to include sufficient patients that suffer from the same non-infectious complication in randomized or observational studies. Management of non-infections complications in PAD therefore follows standardized treatment that is already implemented for other, similar auto-immune disease. For example, in the case of enteropathy and GLILD, treatment that is effective for respectively IBD or sarcoidosis are often used.[29,35]

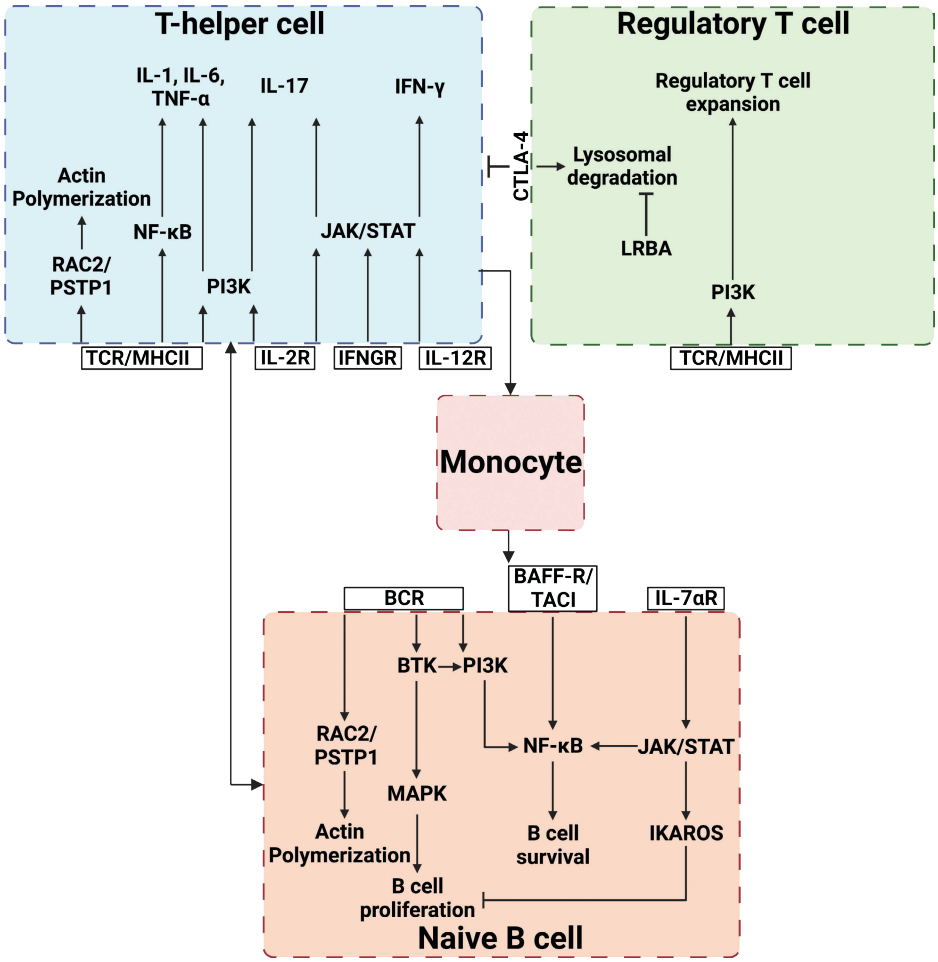
Genetic landscape of PAD

Monogenetic causes of PAD have long been suspected to exist but have not been proven until fairly recently.[36] In the early 1990s, the first monogenetic causes of PAD were identified, when genes related to PAD with a clear Mendelian inheritance pattern, like XLA and CD40L, were successfully sequenced.[37,38] A decade later, in 2003, the first monogenetic cause of CVID was found when autosomal recessive pathogenic variants in ICOS were uncovered.[39] However, since the introduction of next-generation sequencing (NGS), an ever-increasing amount of PAD related genetic variants have been described and currently variants in >40 genes have been identified that cause PAD.[36,39,40] Furthermore, many genes have been associated to a PAD-like phenotype or are investigated as candidate genes for monogenetic PAD.[36,39] However, a monogenetic cause for PAD is found in only 10-20% of cases when current NGS panels are used.[36,39]

The underlying genetic landscape of PAD is very diverse (Figure 3) and involves genes related to B cell development and maturation (IKAROS, TACI, BTK), as well as genes involved in any of the important immunologic signaling pathways, such as: PI3K signaling, JAK/STAT signaling, NFκB signaling, immune checkpoints (such as CTLA4/LRBA), actin skeleton regulation (RAC2, PSTPIP1).[5,36] These immunologic signaling pathways do not only play an important role in B cell function, but can

also be vital for normal T cell function. This diverse landscape underscores the complexity of the necessary immune cell interactions that lead to B cell maturation and prolonged B cell memory.

Figure 3: *Schematic rendition of molecules and signaling pathways involved in B cell differentiation and B/T cell crosstalk associated with PAD-like phenotypes (created with Biorender).*



T cell functionality in PAD

Apart from the lack of B cell maturation and B cell memory formation that is generally associated with PAD, T cell dysfunction has also been shown to play an important role. This has been shown for patients with specific types of monogenetic PAD-like disease, e.g. monogenetic disease caused by STAT1, STAT3, CTLA-4 and LRBA mutations, but also in CVID patients without monogenetic disease

that suffer from non-infectious complications.[41] This has first been shown in two retrospective cohort studies that found increased effector memory T cells in the blood of CVID patients without monogenetic disease that suffer from non-infectious complications.[42,43] Another study has shown that peripheral blood mononuclear cells of these CVID patients show increased expression of IFN-γ response genes.[44] This has been further explored by another study that has shown that T_{h1} and follicular T cells of these CVID patients produce more IFN-γ and that this increase in IFN-γ is related to an increase in dysregulated $CD21^{low}$ B cells.[45] IFN-γ is a known activator of monocytes which in turn can produce B cell activating factor of the TNF family (BAFF).[46] A relation between BAFF and non-infectious complications has specifically been shown in GLILD patients. Here, an increase in BAFF+ monocytes has been shown, specifically in patients with progressive GLILD. Progression of GLILD also correlated with increased BAFF plasma levels, implicating a relation between increased BAFF and B cell dysregulation.[47] These data implicate that T cell dysregulation plays an important role in the pathogenesis of non-infectious complications in PAD, specifically in CVID.

Research objectives and outline of this thesis

Despite previous efforts there are still important knowledge gaps that prevent optimal diagnosis and management of PAD. First, our understanding of the genetic landscape underlying PAD is still limited. While a clear monogenetic cause is identified in 10 – 20% of PAD patients, the outcome of NGS is difficult to interpret in a significant number of patients. Often, variants of unknown significance are identified and the specific role of affected genes within the immune system is not always known. Second, because PAD is a rare disease, larger cohort studies and randomized clinical trials describing optimal prevention of infectious sequalae and optimal treatment of non-infectious complications are lacking. Third, while pulmonary complications are a serious cause of morbidity and mortality in PAD, only a subgroup of patients is affected by them. Pulmonary screening and therapeutical measures are possibly only needed affected patients. However, predictive risk factors that identify patients at risk for bronchiectasis or progressive GLILD are lacking.

Therefore, this thesis focusses on improving our understanding of (monogenetic) PAD and on finding evidence that could improve the management of infectious and non-infectious complications of PAD. Since T cells play an important role in the pathogenesis of non-infectious complications, we are particularly interested

in T cell functionality in (monogenetic) PAD and in clinical and preclinical assays that can normalize T cell function.

The **first part** of this thesis aims to further unravel the hampered crosstalk between B and T cells in PAD by studying patients with newly discovered genes potentially associated with PAD and the functional T and B cell profiles of patients with a known monogenetic disease. In **chapter 2** we describe a new dominant activating mutation leading to constitutively RAC2, which causes dysfunctional rearrangement of the actin skeleton leading to PAD-like disease. We further characterize this mutation using FACS, a GTP pulldown assay and functional neutrophil assays. In **chapter 3** we describe a new autosomal dominant negative mutation leading to dysfunctional c-Myb, which potentially causes an arrest in B cell development and diminished CD4+ and CD8+ T cell function leading to PAD-like disease. In **chapter 4** we characterize functional T and B cell markers present in the blood and tissue of patients with monogenetic PAD like disease by using FACS and cytometry by time of flight (CyTOF). In **chapter 5** we explore a potential assay that could be used to personalize treatment for non-infectious complications in PAD, like GLILD and enteropathy. In this assay we investigate the relation between clinical response to immunosuppressants and *in vitro* inhibition of T cell proliferation and cytokine production.

The **second** part of this thesis aims to give new insights in the prevention of infectious complications and the management of non-infectious complications in PAD. Lung damage through airway disease or GLILD occurs frequently in PAD and therefore pulmonary screening and adequate antimicrobial treatment are the corner stone of clinical management of PAD. However, it is currently unclear which PAD patients are at risk for developing pulmonary damage. We investigate this in **chapter 6**, using a retrospective cohort that was frequently screened for pulmonary damage with CT. Moreover, it is currently unclear if IRT is more effective than prophylactic antibiotics as infection prevention measure in patients with less severe PAD. Therefore, we compare the efficacy and safety of prophylactic antibiotics and IRT in patients with IgSD and/or SPAD in a randomized clinical trial in **chapter 7.**

GLILD is described as a heterogeneous disease with a variable course. GLILD lesions remain stable in some patients, while in other patients GLILD lesions are progressive and cause severe tissue damage. In **chapter 8** we further characterize GLILD, describe the natural course of the disease and investigate

risk factors for disease progression, using a retrospective and prospective cohort study. Moreover, evidence-based treatment guidelines are lacking for GLILD. We therefore performed a systematic review of available literature describing clinical outcomes of GLILD treatment and synthesized the outcomes of different treatment regimens in **chapter 9**. Corticosteroid monotherapy is frequently applied in the clinic, but evidence in literature was found to be lacking. We therefore investigated the efficacy and safety of corticosteroid monotherapy in **chapter 10**.

Finally, HLH is a rare complication of PAD and is challenging to diagnose. Completing all assessments to acquire 5/8 positive diagnostic criteria can be time consuming. We therefore explored a minimal parameter set for HLH diagnosis in **chapter 11,** by using two independent retrospective cohorts as discovery and validation cohort.

In **chapter 12** we describe the conclusions, recommendations and future directives provided by this thesis that can further improve clinical outcomes for PAD patients.

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PART I:

Diagnosis and pathophysiology of PAD

CHAPTER 2

A dominant activating RAC2 variant associated with immunodeficiency and pulmonary disease

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To the editor:

Combined immunodeficiencies (CID) include combined defects of lymphoid cell development and function. We identified a previously reported heterozygous c.184G>A (pE62K) variant in the *RAC2* gene, in a family with three CID affected members.[1]

RAC2 is part of the small Rho GTPase superfamily and is specifically expressed in hematopoietic cells. RAC2 is involved in ROS production through NADPH activation and f-actin remodeling, and is activated upon GTP binding.[2] The GTP/GDP exchange is catalyzed respectively by guanine exchange factors (GEFs) for GTP binding and GTPase activating proteins (GAPs) for GTP hydrolysis.[2] Normal activity of RAC2 and other small Rho GTPases were shown to be crucial for neutrophil and macrophage motility in larval zebrafish and for normal lymphocyte development in mice.[3,4] In humans, earlier loss of function mutations have been described (c.169G>A (p.D57N), c.167G>A (p.W56X) that cause reduced chemotaxis and ROS production in neutrophils and sometimes abnormal lymphocyte numbers.[5,6]

Recently, activating RAC2 variants were described such as the c.184G>A (pE62K) variant, previously reported by Hsu *et al.* in three unrelated CID patients. Here, decreased GTP hydrolysis relayed an increase in active RAC2 leading to severe lymphopenia and neutrophil dysfunction.[1] They replicated these findings in cell culture-based experiments and mouse models and showed upregulated signaling that resulted in increased ROS production and polymerized actin.[1] We report on three related patients with a similar phenotype that carry this RAC2-E62K variant, and elaborate on the molecular changes.

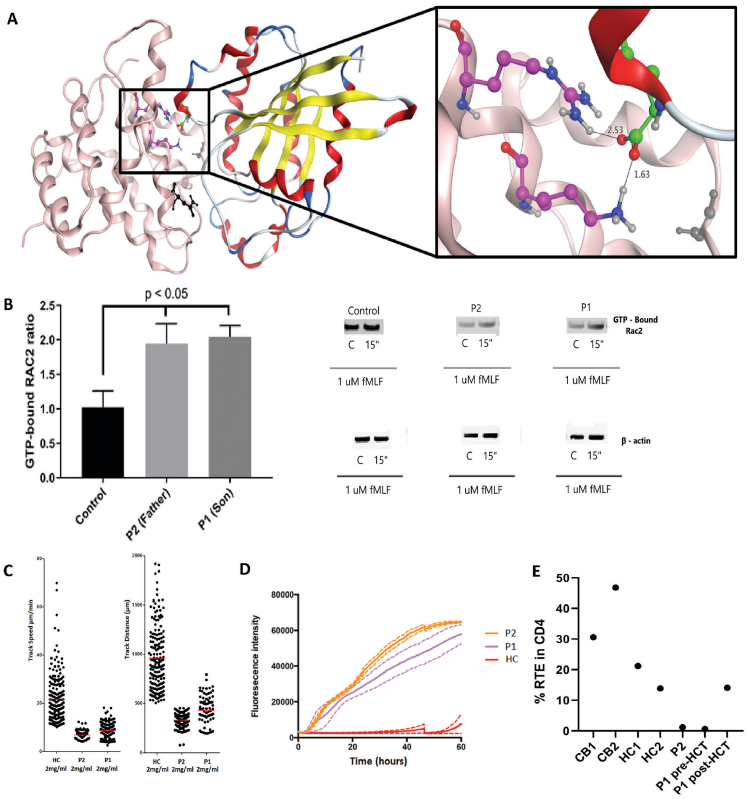
The affected members were a 1-year old (P1), his father (P2) and grandfather (P3). P1 had suffered from respiratory tract infections (RTIs) requiring treatment with oral antibiotics. P2 and P3 had suffered from similar recurrent RTIs causing pulmonary damage leading to end-stage pulmonary failure for which they both required lung transplantation; P3 passed away during this procedure. Lymphocyte phenotyping of P1 and P2 showed T-cell and B-cell lymphopenia with high relative percentages of effector/memory T-cells (Supplementary Table 1), without bone marrow abnormalities. This differed from previously encountered *RAC2* mutations, which did not present with an absolute CID nor with end-stage pulmonary failure.[5,6]

NGS-based immunodeficiency panel analysis showed a heterozygous variant c. 184G>A in *RAC2* causing a glutamic acid to lysine substitution at position 62 (p.E62K). Given the progressive disease course of P2 and P3, we performed hematopoietic cell transplantation (HCT) with unrelated cord blood after myeloablative conditioning in P1. Neutrophils reconstituted >1,8 * 10^9/mL after 23 days and CD4 T-cells reconstituted >100 * 10^6/mL after six months. Absolute CD4, CD8 and B-cell numbers normalized one year after transplant, during which P1 suffered from CMV and HHV6 reactivation and acute graft versus host (GvHD) of the gut and acute chronic GvHD of the skin which was accompanied by increased numbers of CD4 and CD8 effector/memory cells. He is being treated with prednisone, tacrolimus and mycophenolate which sufficiently suppresses his skin GvHD and his T-cell subsets are normalizing. He currently suffers from frequent infections and his pulmonary function test results are suboptimal. P2 is clinically well after pulmonary transplantation with normalization of his pulmonary function test and a reduction of his RTIs. He is still severely immunocompromised with CD4 counts <100 * 10^6/mL and lymphocyte counts <0.5 * 10^9/mL.

We used RAC2 crystal structures (PDB IDs 2W2V, 2W2T) and HADDOCK to structurally investigate variant E62K. We found no direct effect on GTP binding with minimal atom-atom distances >1 nm and hence analysed Rac2/RacGAP1 interaction.[7] We observed two salt-bridges in the highest-ranked docked RAC2-RacGAP1 structure that disappear upon introduction of the mutation (figure S1A). This could hamper GAP binding to RAC2, which would reduce GTP hydrolysis.

To confirm this, we measured relative GTP-bound RAC2 levels in patient neutrophils.[5] We immunoprecipitated GTP-bound RAC2 with PAK-loaded beads and found that only after fMLF stimulation there was a 2-fold increase of GTP-bound RAC2 in patient neutrophils (Figure S1B). Data shown is from normalized data, obtained after merging three controlled experiments that each included healthy donor and patient data; examples of individual blots are shown.

Figure 1: *E62K potentially hampers RAC2/RacGAP1 interaction, causing constitutively active RAC2.*
S1A. Docked model of the RAC2-RacGAP1 complex; the right panel zooms in on the interface region that includes residue E62 of RAC2 (highlighted in ball-and-stick representation, carbon atoms in green). Within the complex, RAC2 is the protein on the right-hand side, RacGAP1 is given in pink. Two candidates for salt-bridge formation with E62 (K423 and R427 of RacGAP1) are shown in ball-and-stick representation with carbons in magenta; atomic distances are indicated in Å. Other key residues for RacGAP1 binding (N495, black; G388, grey) are also highlighted.
S1B. GTP-bound RAC2 ratio, normalized for GTP-bound RAC2 at t=0, both father and son have significantly more GTP-bound RAC2 after 15 seconds of stimulation with fMLF (1 mM, one-way ANOVA, p<0,05) when compared to neutrophils of one healthy donor. This effect was replicated twice after which the average GTP-bound ratios were compared.
S1C. Chemotaxis of affected neutrophils compared to a healthy control. Both the track speed (left) and distance (right) of patient neutrophils is diminished when compared to healthy controls when stimulated with fMLF in varying concentrations of fibrin gel (concentration under y axis).
S1D. Fluorescence after incubation of GFP-expressing S. aureus with healthy and patient neutrophils. Patient neutrophils are incapable of killing S. aureus resulting in immediate colonization at an S. aureus concentration of 5 x 10(S4A), at a concentration of 10 x 10 the healthy neutrophils can also not prevent S. aureus from growing.
S1E. Number of recent thymic emigrants in father (P2) and son (P1) compared to adult healthy controls (HC) and cord blood of two new-borns (CB) in the CD4 T-cell population gated as CD3+, CD4+, CD8-, CD45RA+, CD27+, CD25+, CD62L-, CD31-, and CD21+.



To assess neutrophil function, we cultured neutrophils in 3D fibrin matrices.[8] Migratory speed was reduced by 50% (Figure S1C) and when cultured with GFP-expressing *S. aureus* we found reduced bacterial killing, triggering outgrowth of *S. aureus* at a concentration of 5 x 10^6/mL and a multiplicity of infection of one (Figure S1D).

Finally, we investigated the suppressed T-cell numbers in RAC2-E62K patients, which could be caused by decreased thymic emigration, because the patients expressed no bone marrow abnormalities, and normal thymic populations and outgrowth in RAC2-E62K mice were found[1]. The number of CD4 T-cells expressing recent thymic emigrant (RTE) markers were severely reduced in both patients, but partially restored in P1 after HCT (Figure S1E). These results could be biased by relative abundance of effector memory T-cells that were previously shown in RAC2 patients and in P2, however, P1 had relatively normal naive and effector/memory numbers before transplantation and only showed an expansion of memory T-cells after transplant. Thus, the lymphopenia in RAC2-E62K patients could involve disturbances in RAC2 signaling-mediated chemotactic function of (precursor) T-cells.

This additional data supports that deficient GAP binding is the underlying cause for the clinical phenotype of the recently reported RAC2-E62K variant[1]. It corroborates that internal non-catalyzed GTP hydrolysis is sufficient to regulate RAC2 activation under unstimulated conditions. When patient neutrophils become activated, however, GAPs cannot bind efficiently and consequently GTP-bound RAC2 increases. The same mechanism might be relevant for other *RAC2* variants at the interface with GAP binding, supported by the fact that patients with a P34H mutation show a similar phenotype.[7,9]

The increase in GTP-bound RAC2 might impede physiological f-actin polymerization, which could cause neutrophilic dysfunction, and possibly, the underlying lymphopenia by hampering thymic emigration. This is reminiscent of phenotypes of WASp and DOCK8 variant cells, which are both effectors of the small GTPase Cdc42.[10]

In conclusion, we report additional clinical and molecular findings on the recently discovered dominant activating RAC2-E62K variant, which we found in three related patients. Our data support that the RAC2-E62K variant leads to more GTP-bound, active RAC2 by hampered GAP binding.

Authorship contributions

BS, CK, MD, DG, SN, MB, MG, LK, AB, HW and MT conceived and designed experiments.
BS, FV, CK, MD, PL, LC and MT performed the experiments.
BS, CK, MD, DG, MB, and LK analyzed the data.
JM, SN, HL, JJB, CL, AB and LC provided clinical care.
SN, MB, MG, LK, AB and HW provided reagents, materials and analysis tools.
BS, MB, HRW and LK wrote and edited the manuscript.
All authors read and approved the final manuscript.

Acknowledgements

We thank Ninke Nieuwenhuis and Willemien Zuilhof for discussions on and reviewing of previous studies on RAC2-GAP interactions. We also would like to thank our patients for providing us with the information and samples needed to perform this study.

Disclosure of Conflict of Interest

None to disclose

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SUPPLEMENTAL TABLES

Supplemental table 1: *CDC and immunophenotyping data of P1 and P2 showing a combined immunodeficiency phenotype.*

	P2	P1	Adult reference values	Age-matched pediatric reference values
Hemoglobin (mmol/L)	8	7.1	8.6 – 10.7	7.4 – 8.7
Platelets (10^9/L)	113	223	150 – 450	150 – 450
Leukocytes (10^9/L)	3.1	3.2	4.0 – 10.0	5.5 – 15.5
Neutrophils (10^9/L)	2.58	1.57	1.6 – 8.3	1.5 – 8.5
Lymphocytes (10^9/L)	0.19	1.01	0.8 – 4.0	2.0 – 8.0
T-cells	117	480	700 – 900	1800 – 5900
CD4	30	242	560 – 1067	1902 – 2977
CD4 Naive (%)	6.0	54.3	49.4 – 71.9	55.6 – 75.8
CD4 Effector/Memory (%)	94.0	45.7	27.3 – 49.8	24.0 – 43.4
Active CD4 (%)	2.4	15.9	0.3 – 1.0	0.3 – 1.8
CD8	82	217	216 – 499	667 – 1473
CD8 Naive (%)	3.2	46.3	48.6 -87.5	57.0 – 83.7
CD8 Effector/Memory (%)	96.8	53.7	11.7 – 42.9	13.4 – 29.9
Active CD8 (%)	3.5	39.9	0.9 – 4.2	1.1 – 3.2
B-cells	55	229	114 – 436	871 – 1553
NK-cells	17	170	9 – 24	100 - 1100
IgM (g/L)	0.42	1.3	0.4 – 2.3	0.1 – 0.87
IgA (g/L)	0.73	0.5	0.7 – 4.0	0.19 – 1.1
IgG (g/L)	6.22	10	7.0 – 16.0	2.6 – 13.9

CHAPTER 3

Heterozygous variants in the DNA-binding domain of c-Myb may affect normal B/T cell development

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Dear Editor,

Inborn errors of immunity (IEI) are serious immunological disorders characterized by aberrant development, proliferation, regulation or function of immune cells.[1] The introduction of next-generation sequencing has facilitated the discovery of new causes of IEI.[2] Already >400 IEI related genes have been identified, and classified according to the affected component of the immune system.[1]

The MYB proto-oncogene (*MYB*) has previously been identified in both *in vitro* and *in vivo* models as a key player in transcription factor networks involved in stem cell and hematopoietic cell development, but has not yet been related to IEI.[3] While homozygous null variants of *MYB* in mice have been shown to be lethal, heterozygous, temporal and local null models of the DNA binding domain exon in mice and human cell lines have shown that its product, the c-Myb transcription factor, is crucial for pro- and pre-B cell differentiation by controlling the expression of IL-7 receptor-α, and Rag and the initiation of survival signals.[3,4] Moreover, c-Myb is vital for thymocyte development and regulates two distinct pathways in mature CD4+ and CD8+ cells; in CD4+ cells it is involved in Th2 development, through GATA3 regulation, while it regulates central memory stemness in CD8+ cells, through *Tcf7* and *Bcl2* upregulation and *Zeb2* repression (Figure 1A).[5,6]

Here, we describe two patients who presented with a combined immunodeficiency that progressed into severe bone marrow dysfunction with distinct *de novo MYB* heterozygous DNA binding domain variants.

Participants provided written informed consent for institutional review board–approved studies in the Netherlands (National PID study, METC: NL40331.078) and Canada (Care4Rare).

SNP-array copy number variant (CNV) profiling and analysis of regions of homozygosity were performed on DNA isolated from peripheral blood according to standard procedures, using the Infinium Human CytoSNP-850K v1.0 BeadChip (Illumina) and the Nexus software v7 (BioDiscovery) with Human genome build Feb. 2009 GRCh37/hg19.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood according to standardized protocols and stored in liquid nitrogen. After thawing, T cells were isolated with CD3+ magnetic beads and MS Columns (Miltenyi Biotec)

and seeded in 24-wells plates at a 1*10^6/mL density and cultured for 0, 24, 72 & 120 hours with CD3/CD28 Dynabeads (Thermo Fisher Scientific).

RNA was isolated with the RNeasy Mini Kit (Qiagen) and cDNA was obtained, using the RevertAid First Strand Kit (Thermo Fisher Scientific). Primers selected from earlier publications (Supplementary Table 1, Integrated DNA Technologies) were used for real-time PCR using the SYBR Select Master Mix (Thermo Fisher Scientific). [7–9]

Antibodies used for flow cytometry are listed in Supplementary Table 2. For intracellular staining of c-Myb, Tcf7, and Zeb2, cells were fixed and permeabilized (eBioscience, 00–5524). BDFortessa II (BD Biosciences) was used for flow cytometry acquisition. Samples were analyzed with FlowJo software (TreeStar). Telomere length was measured by a 2-panel assay by flow fish technology at RepeatDx (https://repeatdx.com).

Results were analyzed using the two-tailed students-t test in GraphPad Prism v9.

A 22-year-old male of Dutch origin with a history of tetralogy of Fallot presented with recurrent lower tract infections at the age of 3 years. Laboratory test results showed reduced IgG and IgM and absent peripheral B cells. Fourteen B cell disorder associated genes were analyzed and no disease-associated variants were identified. He was diagnosed with common variable immunodeficiency (CVID) and treated with intravenous immunoglobulin replacement therapy (IgRT).

At the age of 9 years, he developed a slowly progressive thrombocytopenia and at the age of 19 years, bone marrow examination showed normal cellularity with dysplastic features in all lineages. Furthermore, he developed oral aphthous lesions and leucoplakia. Immunological investigations showed monocytopenia, neutropenia, reduced NK cells and low fractions of naive CD8+ T cells with impaired proliferation upon antigen stimulation and a reduced V-beta repertoire (Table 1).

Table 1: *Baseline characteristics, CBC at onset, immunoglobulins at onset, immunophenotyping at onset and functional tests of both MYB cases.*

	Case 1	Case 2
Baseline Characteristics		
Sex	Male	Male
Age at presentation	3 years	3 years
Current Age	23 years	11 years
MYB variant	c.545A>G	c.383A>G
CID classification	$T^+B^-NK^-$	$T^+B^-NK^+$
CBC		
Hemoglobin (mmol/L)	8.1	8.3
Platelets (x10^9/L)	326	148
Leukocytes (x10^9/L)	4.6	2.1
Neutrophils (x10^9/L)	1.32	2.1
Lymphocytes (x10^9/L)	2.39	0.2
Immunoglobulins (Ig)		
IgM (g/L)	<0.04	0.25
IgA (g/L)	0.55	< 0.0667
IgG (g/L)	5.19	0.34
Immunophenotyping		
CD3+ (x10^9/L)	2.3	0.726
CD3+CD4+ (x10^9/L)	1.2	0.440
CD3+CD8+ (x10^9/L)	1.1	0.165
CD19+ (x10^9/L)	0	0.011
CD56+ (x10^9/L)	0.03	0.319
Functional Tests		
TREC	NA	Absent
v-beta repertoire	Reduced	Normal
T cell proliferation	Reduced for CD8+	Normal
Lymphocytic telomeres	<p2	NA

CBC = Complete Blood Count, CID = Combined Immunodeficiency, Ig = Immunoglobulin, TREC = T-cell receptor excision circles

Trio exome sequencing (ES) demonstrated a *de novo* heterozygous c.545A>G variant in *MYB* causing a p.(Lys182Arg) substitution within the DNA binding domain of c-Myb. This variant had never been reported in gnomAD and prediction software predicted the variant to be damaging. Hence the variant was classified as a variant of unknown significance (VUS) and was submitted to the Matchmaker Exchange in order to identify other unrelated individuals with rare variants in the same gene and overlapping phenotypes.[10] Other pathogenic variants in known genes associated

with primary immunodeficiencies were not found and SNP-array analysis showed a normal array profile without indications for pathogenic CNVs in 22q11.

T cell phenotyping showed decreased naive T cell fractions and increased TEMRA fractions in the patient compared to healthy controls and age-adjusted reference values (Figure 1B). Real-time RT PCR showed that *c-Myb, Tcf7,* and *Bcl2* expression was comparable to that of healthy controls in unstimulated T cells, while *Zeb2* expression was increased 11-fold (Figure 1C, $p<0.01$). After 72 hours of CD3/CD28 stimulation, peak *c-Myb* and *Bcl2* expression was reached, while *Tcf7* expression and *Zeb2* repression peaked after 120 hours (Supplementary Figure 1). There was a trend towards lower peak expression of c-*Myb, Tcf7 and Bcl2* ($p=0.19$, $p=0.19$ & $p=0.21$) in patient cells, while peak repression of *Zeb2* was not significantly different ($p=0.42$) (Figure 1D). Protein analyses using flow cytometry for c-Myb, Tcf7 and Zeb2 showed similar results (Supplementary Figure 2), and additionally indicated that expression of c-Myb and Tcf7 was especially hampered in the patient's CD8+ T cells ($p<0.01$ & $p<0.01$) (Figure 1E-G).

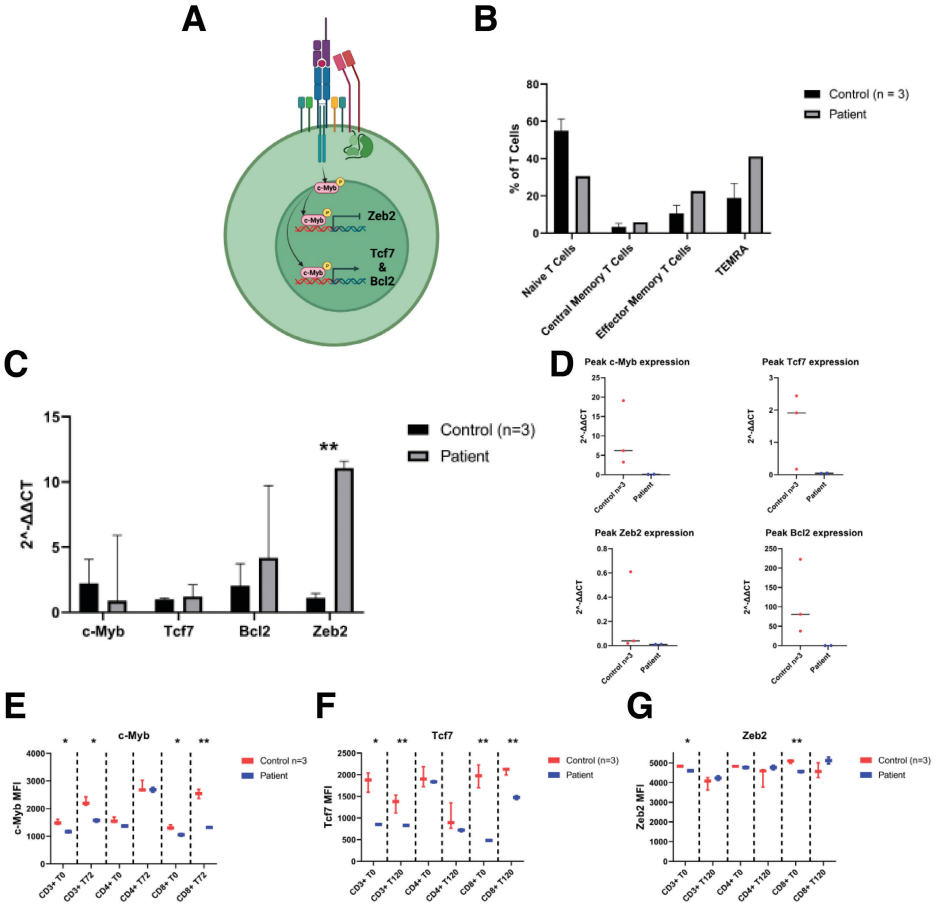
A telomere length assay demonstrated extremely short lymphocytic telomeres (<<p2).[11] Based on the combination of clinical features (immunodeficiency, bone marrow failure, leucoplakia, congenital heart defect) a non-classical telomeropathy was suspected. Trio ES and Mytomycin C culture were unremarkable, aside from a shared heterozygous variant in *CTC1* ($c.3136_{del}$) between the father and the patient.

Due to significant thrombocytopenia, elective hematopoietic stem cell transplantation (HSCT) was considered for progressive bone marrow failure at age 21. However, since his thrombocytopenia was responsive to a course of dexamethasone and CBC was stable, HSCT has been postponed thus far.

An unrelated patient of French-Canadian origin presented with recurrent otitis media and invasive pneumococcal disease (sepsis, pneumonia and empyema due to *Streptococcus pneumoniae*, serotype 19A) at the age of 3 years. Immunological work up revealed profound hypogammaglobulinemia with absent vaccine responses, including pneumococcal responses (14 serotypes tested). He had T cell lymphocytopenia and near absence of CD19 B cells. Moreover, TRECs were undetectable with decreased recent thymic emigrant naive T cells. V-beta repertoire, as well as T cell proliferation studies (PHA/OKT3), were normal. Limited targeted genetic testing including a SCID-B negative panel and testing for *GATA2* deficiency was negative. He was treated with intravenous IgRT. He subsequently developed polyarthritis and rubella positive necrotizing granulomatous

dermatitis.[12] Bone marrow examination showed normocellular bone marrow with active trilinear hematopoiesis. Given his combined immunodeficiency syndrome, he underwent a successful matched unrelated HSCT at age 6.

Figure 1: c-Myb and its downstream targets are differently expressed in two technical replicates of T cells carrying the p.Lys182Arg variant. *Results were analyzed with two-tailed student-t tests, *p<0.05 **p<0.01. (a) In activated mice CD8+ T cells, transcription factor c-Myb has been found to increase Tcf7 & Bcl2 expression, while repressing Zeb2 expression through competitive binding to the promoter[6] (b) Proportions of CD3+ subsets in the patient vs 3 healthy controls showing that the patient has reduced naive CD3+ fractions and increased TEMRA fractions.(c) Expression of levels of c-Myb, Tcf7, Bcl2 and Zeb2 in unstimulated T cells. 2^-ΔΔCt values were obtained using the mean ΔCt values of the healthy controls. Zeb2 expression is higher in patient samples. (d) Peak c-Myb, Tcf7 and Bcl2 expression and Zeb2 repression in T cells after stimulation. 2^-ΔΔCt values were obtained using the mean ΔCt values of unstimulated T cells. Patient cells show a trend towards decreased c-Myb, Tcf7, and Bcl2 expression and similar Zeb2 expression. (e-g) Mean fluorescence intensity (MFI) of c-Myb, Tcf7, and Zeb2 in CD3+, CD4+ and CD8+ cells. Peak protein expression of both c-Myb and Tcf7 was lower in CD3+ and CD8+ cells, but not in CD4+ cells.*



Trio ES analysis detected a *de novo* heterozygous DNA binding domain missense variant c.383A>G p.(Lys128Arg)] of *MYB*. This variant was at a conserved nucleotide (GERP 5.81) and also had not been previously reported in presumed healthy controls. *In silico* analysis programs predicted that the missense had damaging impact on protein function and/or structure (CADD 29, SIFT 0, PolyPhen 0.996, Vest3 0.914).

Here we report on two patients with heterozygous DNA binding domain variants of c-Myb, presenting with combined immunodeficiency like symptoms and bone marrow failure. Similar to *in vivo* models these patients both showed severe B cell lymphocytopenia and hypogammaglobulinemia.

Moreover, like earlier research showing that deletions in c-Myb cause defects in CD8+ cell signaling, we here show similar defects in CD8+ cells carrying the p.(Lys182Arg) variant.[5] However, post-stimulation peak Zeb2 repression was not significantly reduced. Instead, Zeb2 mRNA expression was significantly higher in unstimulated p.(Lys182Arg) T cells while protein expression was lower in the unstimulated condition. This could suggest that the p.(Lys182Arg) does not influence peak Zeb2 repression, but does play a role in long term Zeb2 repression, which is in turn counteracted by Zeb2 specific translational control. In non-small-lung cancer, specific translational control of Zeb2 has been shown through microRNA-342-3p binding.[13]

Previously, one other patient with a more extensive phenotype consisting of immunodeficiency, progressive bone marrow failure, short stature and dysmorphic facial features and a heterozygous 3.4Mb deletion of chromosome 6, including *MYB,* has been described further implicating the association between *MYB*, IEI, and bone marrow failure.[14]

Furthermore, deficiency of proteins that express MYB-like DNA binding domains, telomeric repeat binding factor 1 and 2 (TERF1/2), have been implicated in telomere disease biology.[15] TERF1/2 protect telomeres from damage, inhibit the lengthening of telomeres and monitor the cell-aging process through inhibition of telomerase activity by binding to the TTAGGG repeats .[15] Pathogenic variation in TERF1/2 has been shown to cause a non-classical telomeropathy associated with severe aplastic anemia.[11]

DNA binding domain variants might lead to potential polymorphic effects of c-Myb interactions with the binding sites of TERF1/2, triggering competition for the TTAGGG binding site, hampering telomeric TERF1/2 binding, leaving the telomeres unprotected.[15]

However, without *in silico*, *in vitro* and specific *in vivo* models, to prove the effect of heterozygous DNA binding domain variants of c-Myb, these putative mechanisms remain highly speculative and the criteria for novel IEI, outlined by the IUIS consortium, have not yet been fulfilled.[1] It is imperative that in the future such models are developed, to further investigate the role of DNA binding domain variants of c-MYB in IEI, and telomere dynamics.

In conclusion, heterozygous variants in the DNA-binding domain of c-Myb should be further investigated as a possible candidate for IEI, since they are potentially related to defects in B and T cell development, telomeropathy, and consecutive myelodysplasia.

Acknowledgements:
Portions of this work were performed under the Care4Rare Canada Consortium funded by Genome Canada and the Ontario Genomics Institute (OGI-147), the Canadian Institutes of Health Research, Ontario Research Fund, Genome Alberta, Genome British Columbia, Genome Quebec, and Children's Hospital of Eastern Ontario Foundation.

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CHAPTER 4

Functional T cell and B cell profiles can distinguish between primary immune regulatory disorders.

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ABSTRACT

Background: Primary immune regulatory disorders (PIRD) are characterized by immune-mediated diseases resulting from inborn errors of immunity. Despite overlapping features, distinct immunological profiles have been described for specific PIRD subtypes, such as APDS, STAT1GOF, STAT3-HIES, and STAT3GOF, yet have not been linked to tissue pathology such as enteropathy, complicating development of targeted treatment in blood and inflamed intestinal tissue.

Objective: This study aimed to differentiate immunological profiles of APDS, STAT1GOF, STAT3-HIES, and STAT3GOF.

Methods: Peripheral blood mononuclear cells from PIRD patients (n = 11) and healthy controls (n = 10) were analyzed using cytometry by time of flight (CyTOF) and fluorescence activated cell sorting (FACS). Inflamed intestinal tissue from 4 PIRD patients and 4 uninflamed controls was spatially profiled using imaging mass cytometry (IMC).

Results: The study included 3 APDS, 4 STAT1GOF, 3 STAT3-HIES, and 3 STAT3GOF patients. CyTOF analyses revealed that APDS and STAT3GOF patients had increased proportions of CD4+ and CD8+ central memory T cells, while STAT1GOF and STAT3-HIES had higher levels of CD4+ naive T cells. FACS analyses using T cell panels for homing, activation, exhaustion and intracellular cytokine production enabled further discrimination of PIRD subgroups. IMC analyses demonstrated reduced intraepithelial T cell numbers with reduced differentiation and inflammation markers, such as cytokines, in all PIRD compared to uninflamed controls.

Conclusion: The distinct subgroups of PIRD may be differentiated based on T cell subset markers in peripheral blood. Conversely, T cells may play a lesser role in intestinal tissue inflammation in PIRD than previously assumed.

Clinical Implication: Immunophenotype can potentially distinguish PIRD subgroups, although further confirmation in a larger prospective cohort study is warranted. Additionally, these findings suggest that T cells might play a smaller role in the local pathogenesis of intestinal inflammation in PIRD then previously assumed.

Keywords: Primary immune deficiency, innate errors of immunity, imaging mass cytometry, CyTOF, immune dysregulation, FACS

ABBREVIATIONS

APDS	Activated phosphoinositide 3-kinase delta syndrome
CyTOF	Cytometry by time of flight
FACS	Fluorescence activated cell sorting
IEI	Inborn errors of immunity
IMC	Imaging mass cytometry
PBMC	Peripheral blood mononuclear cells
PIRD	Primary immune regulatory disorders
STAT1GOF	STAT1 gain-of-function
STAT3GOF	STAT3 gain-of-function
STAT3-HIES	STAT3 dominant negative hyper IgE syndrome
T_{h17}	T helper 17
UMCU	University Medical Center in Utrecht

INTRODUCTION

Patients with inborn errors of immunity (IEI) often suffer from opportunistic infections or aberrant responses to infections. In such cases, the disease is classified as primary immunodeficiency disorder.[1–3] Certain patients with IEI exhibit immune mediated disease characterized by auto-inflammation and auto-immunity and the condition is classified as a primary immune regulatory disorder (PIRD).[2,4,5] Besides immune mediated diseases, PIRD patients often present with an antibody deficiency-like phenotype leading to the requirement of immunoglobulin replacement therapy.[2,6–9] With the advent of next-generation sequencing, new PIRD related genetic variants are discovered at a rapid pace. Currently, 129 PIRD related genes have been described, accounting for approximately 30% of all known IEI related genes, which makes PIRD one of the most prevalent disorders among IEI patients suffering from severe morbidity and mortality.[10–12]

Activating PI3K mutations, leading to activated phosphoinositide 3-kinase delta syndrome (APDS), STAT1 gain-of-function (STAT1GOF), STAT3 gain-of-function (STAT3GOF) and potentially STAT3 dominant negative hyper IgE syndrome (STAT3-HIES) are all examples of PIRD that are characterized by B cell and sometimes T cell aberrancies and severe immune mediated pathology.[2,13–16] B cell aberrancies are observed in all conditions and can manifest as various forms of antibody deficiency, ranging from mild hypogammaglobulinemia to specific polysaccharide antibody deficiency or a common variable immunodeficiency-like phenotype. T cell aberrancies, primarily observed in APDS, is characterized by a reduction in CD4+ (naïve) T cells.[14,16–25] In APDS, STAT1GOF and STAT3GOF, approximately 35% to 95% of described patients exhibited at least one immune-mediated comorbidity.[23] Auto-immune cytopenias, endocrinopathies, glomerulonephritis, interstitial lung disease, lymphoproliferation, and enteropathy have been described as the most prevalent f immune mediated pathologies. [14,16–26]

STAT3-HIES is a monogenetic disease showing partial overlap with symptoms of STAT1GOF.[27–30] In both diseases (STAT1GOF: 98%, STAT3-HIES: 70%), patients suffer from chronic mucocutaneous candidiasis.[31,32] Moreover, approximately half of the patients exhibit an increased susceptibility to lower respiratory tract infections and a lack of T helper 17 (T_{h17}) cells has been described.[24,27,28,31] Additionally, clinical signs of enteropathy (60%) and esophageal eosinophilic infiltration (15%) has been described inf STAT3-HIES patients.[33]

Enteropathy is one of the most prevalent immune-mediated pathologies in PIRD subgroups but its mechanisms are poorly understood. It is diagnosed when gastrointestinal symptoms occur without an infectious cause[34] and presents as acute IBD-like inflammation but can also be more chronic in nature. Patients with enteropathy may experience diarrhea, bloating, reflux and pain, leading to protein-loss and malnutrition.[35,36] Research into immunological markers in the gut is scarce and has not yet been performed in most of these PIRD subgroups. In primary antibody deficiency without a monogenetic cause, enteropathy is associated with lymphocytic infiltrates enriched in T cells and diminished in plasma cells, but studies that further investigate T cell subtypes and function are lacking.[34,37]

There are currently no evidence-based treatment guidelines for enteropathy.[34] Previously, the efficacy of local and systemic corticosteroids, azathioprine, TNFα blockade and vedolizumab have been described in small cohorts of PAD patients with limited success.[34] More recently, pathway specific inhibitors, like JAK inhibitors and PI3K inhibitors have been used to treat immune mediated pathologies in PIRD, however their specific efficacy for enteropathy is scarcely described.[38–41] Moreover, pathway specific inhibitors have been associated with more infectious complications.[40,41] New and safe therapeutic targets specific for PIRD associated enteropathy are therefore required, underlining the need for a clear understanding of immune-mediated pathogenesis.

Although distinct immunological profiles have been described in blood between these PIRD subgroups, the clinical and immunologic profiles of individual patients can be diverse and overlap between the different diseases.[22,23,28,42] Moreover, immunological profiles of various PIRD subgroups have rarely been compared in one study and it is currently unknown whether these diseases could be distinguished based on immunological profiles alone. Of note, it remains uncertain whether immunological phenotypes in the blood, resemble tissue and whether specific subpopulations of T or B cells exist that could be a therapeutic target for enteropathy in these patients

Therefore, this study aims to distinguish the distinct immunological profiles of APDS, STAT1GOF, ADHIES and STAT3GOF by using extensive fluorescence-activated cell sorting (FACS) and cytometry by time of flight (CyTOF) to generate immunological profiles in blood and inflamed tissue.

MATERIALS AND METHODS

Ethics Statement

The Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam, The Netherlands (METC: 2013–026) provided ethical approval for all patients in this study. The technical committee for biological sample collection of the University Medical Center in Utrecht (UMCU), The Netherlands (TCbio: F10P50) provided ethical approval for all healthy controls in this study. Written informed consent was obtained from all patients and controls according to the Declaration of Helsinki. Human tissue that was used for validation was approved by the Medical Research Ethics Committee of Utrecht, the Netherlands (METC 20-102) and Dutch Nationwide Pathology Databank (PALGA 2020-53).

Blood Donors and PBMC isolation

Blood samples of patients with a pathogenic or likely pathogenic variant that caused STAT1GOF, STAT3-HIES, APDS or STAT3GOF were acquired from the UMCU, and the Erasmus Medical Center. Blood of healthy controls was acquired from healthy UMCU personnel via the Mini Donor Service (TCbio: F10P50). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation (GE Healthcare-Biosciences, AB), and stored at -180 °C in FCS + 10% DMSO (METC: 2013–026) until use.

Tissue Samples

Tissue of patients with a pathogenic or likely pathogenic variant that caused STAT1GOF, STAT3-HIES, APDS or STAT3GOF and who had developed severe enteropathy-like gastrointestinal symptoms, that required a diagnostic endoscopy or colonoscopy was retrospectively included from biobank of the UMCU (n = 4). Biopsies were examined by the Pathology Department of the UMCU. Medication and clinical patient characteristics and demographics during sampling were obtained from electronic patient files.

Antibody Panel and Antibody Conjugation

A detailed overview of the designed 35-plex imaging mass cytometry panel and 22-plex CyTOF panel is provided in **Supplementary Table 1** and **Supplementary Table 2**, respectively. BSA and glycerol free antibodies (100 microgram) were conjugated according to manufacturer's protocol, validated and titrated.

PBMC preparation

PBMCs were thawed and recovered in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine 100 U/ml penicillin-streptomycin, and washed. PBMCs were then further processed for CyTOF or for FACS.

CyTOF staining and barcoding protocol

Cells were rested for 2 hours at 37°C, after which 250.000 cells were transferred into a 96-well V-bottom tissue cultured-treated well plate (Corning, United Kingdom). Cells were fixed with 2% PFA (electron Microscopy Sciences) for 10 minutes at room temperature. Fixed cells were washed with cell staining solution (Fluidigm) and incubated with the surface staining mix for 45 minutes at room temperature. Next, cells were washed with cell staining solution and resuspended in ice-cold methanol (Sigma-Aldrich) and incubated overnight at -20 °C. Cells were Barcoded using the Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm) Formalin-crosslinked and methanol-permeabilized cells were washed with cell staining solution and with the intracellular staining mix for 45 minutes at room temperature. Then, cells were washed with cell staining solution and resuspended in PBS containing Cell-ID Intercalcator (Fluidigm) at a 1:5000 dilution, and incubated overnight at 4°C. Before CyTOF analysis, cells were washed with cell staining solution and resuspended in cell acquisition buffer (Fluidigm).

CyTOF and Data Analysis

The CyTOF Helios and mass cytometer (Fluidigm) were calibrated and operated according to the manufacturer's instructions. Just before sample acquisition, EQ four element calibration beads (Fluidigm) were added to the cell suspension in a 1:10 (v/v) ratio. Next, FCS files were concatenated using the manufacturer's software (Fluidigm). Then the CyTOF Software for Helios and Hyperion systems was used to remove beads and normalize the data. Data was further processed using Cytobank (https://www.cytobank.org). Data was cleaned up and viable cells were gated on the viable channel (195Pt negative cells), and the DNA channels (191Ir and 193Ir positive cells) (**Supplementary Figure 1**).

FACS

For the FACS analysis, cells were plated at 50,000 live cells per extracellular staining condition and 100,000 live cells per intracellular staining conditions. For the B cell panel, the T homing panel and the T_{reg} panel, cells were directly incubated with surface antibodies (Supplementary Table 3) for 30 min at 4 °C and washed. For the T_{reg} panel, cells were then permeabilized with fixation/permeabilization reagent

(eBioscience) for 30 min at 4 °C, washed, and incubated with the intracellular antibodies (Supplementary Table 3). Finally, cells were measured on the LSR Fortessa (BD Biosciences). For the remaining panels, cells were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA, MilliporeSigma) and 1 µg/mL ionomycin (MilliporeSigma) for 4 h at 37 °C with addition of monensin for the panels that measured intracellular cytokine production (Golgistop, BD Biosciences, 1:1500). Next, cells were incubated with surface antibodies for 30 min at 4 °C and washed. Cells were then permeabilized with fixation/permeabilization reagent (eBioscience) for 30 min at 4 °C, washed, and incubated with the intracellular antibodies. Finally, cells were measured on the LSR Fortessa (BD Biosciences). An overview of the three used antibody panels is provided in **Supplementary Table 3.**

Patient tissue samples and tissue microarray

Formalin-fixed paraffin embedded blocks containing duodenum, ileum, jejunum, or colon biopsies of primary immunodeficiency patients with immune dysregulation within the gut were included (n = 13). Tissue microarray (TMA) was composed of various gastro-intestinal tissue biopsies from patients with uninflamed tissue. Patient samples and TMA (4 mM) were cut and transferred onto a Suringpath X-tra adhesive pre-cleaned Micro slide (Leica Biosystems, Amsterdam, the Netherlands). Tissue slides preparation and H&E staining were done by the pathology Department of the University Medical Centre Utrecht (TCbio 19-083).

Region selection

A serial H&E slide of each patient was examined for quality and immune infiltrate in close collaboration with a pathologist specialized in gastroenterology. Samples were only included when they contained an immune infiltrate, without tertiary lymphoid structures or processing artefacts. Three representative regions of four crypts containing homogeneous immune infiltrate were selected per sample.

Tissue preparation and staining

Tissue sections were twice deparaffined for 10 minutes in xylene (Klinipath, Duiven, the Netherlands). Tissue sections were rehydrated using a 99% - 70% alcohol gradient (Sigma-Aldrich, Missouri, USA). Slides were first washed in MilliQ water and then washed with TBST (Sigma-Aldrich, Missouri, USA). For antigen retrieval, slides were placed in a pre-heated 10 mM Tris 1mM EDTA pH 9.5 for 30 minutes at 95 °C (Sigma-Aldrich, Missouri, USA). After cooling, slides were washed in TBST. Glass slides were dried using a paper tissue and the tissue was encircled using a PAP pen (Sigma-Aldrich, Missouri, USA). Tissue blocking was done using TBST

containing 3% BSA and 1:100 Fc receptor blocking solution antibody (Biolegend, San Diego, USA) for 1 hour at room temperature. Samples were stained with CTLA-4 (ab237712, Abcam, Cambridge, United Kingdom) in TBST containing 0,5% BSA (Sigma-Aldrich, Missouri, USA) in a humidified chamber and incubated overnight at 4 °C. After removing the antibody, slides were washed. Metal labeled secondary antibody was incubated for 1 hour at room temperature. Samples were washed in TBST and the metal conjugated antibody mix was prepared in TBST containing 0,5% BSA according to Supplementary table 2. Slides were incubated overnight in a humidified chamber at 4 °C. Slides were washed in TBST and stained with DAPI (1:1000, Sigma-Aldrich, Missouri, USA). After incubation, slides were washed in MilliQ water and dried on air.

Immunofluorescence Microscopy

Slides were imaged with a Zeiss Axio Imager Z1 microscope using a 5x (Zeiss, 420330-9901) and 20x (Zeiss, NA 0.5, 420330-9900) dry objective in combination with a 45 and 49 filter set and a mercury lamp, using a Zeiss Axiocam 503 mono camera. Microscope images were generated in 12 plane Z-stacks with a 10 percent overlap between the tiles using the Zen Pro software (Zeiss, version 2.7.76).

Imaging mass cytometry

After immunofluorescence microscopy**,** samples were washed in MilliQ and were stained with 0.1% toluidine blue (Sigma-Aldrich, Missouri, USA). Slides were washed and stained with DNA Intercalator (1:400, Fluidigm, San Francisco, USA) for 1 hour. Next, slides were washed and dried overnight. Imaging mass cytometry (IMC) was performed on a calibrated Hyperion Imaging system (Fluidigm, San Francisco, USA) coupled to a Helios mass cytometer (Fluidigm, San Francisco, USA). Laser frequency was set to 200 Hz and laser energy between 4-8.

Data analysis and single cell generation

Processing of microscopy and IMC data was performed as described previously.[43,44] Single cell data was extracted from pixel intensities of all channels of all cells in 32-bit unscaled images that were represented in the segmentation maps. R version 4.2.2. was used for single cell data extraction.

Data curation & signal normalization

In Ilastik, the pixel classification workflow was used to identify artefacts in all acquired channels. The following feature was selected: Gaussian Smoothing (σ0.3, 1.0). Single-cell events with a mean probability above 0.1 for predicated noise

were excluded. Next, signal levels for individual image regions were normalized using the median of scaled intensities of all the channels excluding the channels: H3, Ir191, and Ir193.

Statistical analysis

For the CyTOF data we performed clustering analysis using FlowSOM in Cytobank with the following clustering parameters: 20 clustering channels using all cell surface markers; event sampling (equal); clustering (hierarchical consensus); metaclusters (12), clusters (100), iterations (10), seeds (automatic). Clustering analysis of FACS data was performed in R and potentially distinguishing populations were analyzed using one-way ANOVA. The family-wise error rate was controlled using the Holm-Bonferroni method. For IMC, expression values were calculated as median expression of all cells per image. Heatmaps were generated using the pheatmap (RRID: SCR_016418) package in R. Relative expression was compared using one-way ANOVA and $p<0.05$ was considered significant.

RESULTS

Patient Characteristics

Three APDS, four STAT1GOF, three STAT3GOF and three STAT3-HIES patients and ten healthy controls were recruited for this study (Figure 1A). Patient characteristics are described in Table 1. Genetic evaluation using NGS in combination with a PID filter yielded (likely) pathogenic variants 10/13 patients and 1 STAT1GOF and 2 STAT3GOF variants that were initially classified as variant of unknown significance (VUS). The STAT3GOF patients with a VUS showed symptoms typical for PIRD and molecular evidence of STAT3GOF and were therefore reclassified as likely pathogenic. Peripheral blood was available for 11 out of 13 patients and tissue samples were available for two APDS, one STAT1GOF and one STAT3GOF patients (Table 1). None of the patients were actively treated with immunosuppressive therapy before peripheral blood sampling. All APDS patients and two STAT3-HIES patients received IVIG during peripheral blood sampling. One STAT3GOF patient (P12, Table 1) was actively treated with immunosuppressive therapy before tissue sampling.

Table 1: Included patients and their genetic variants

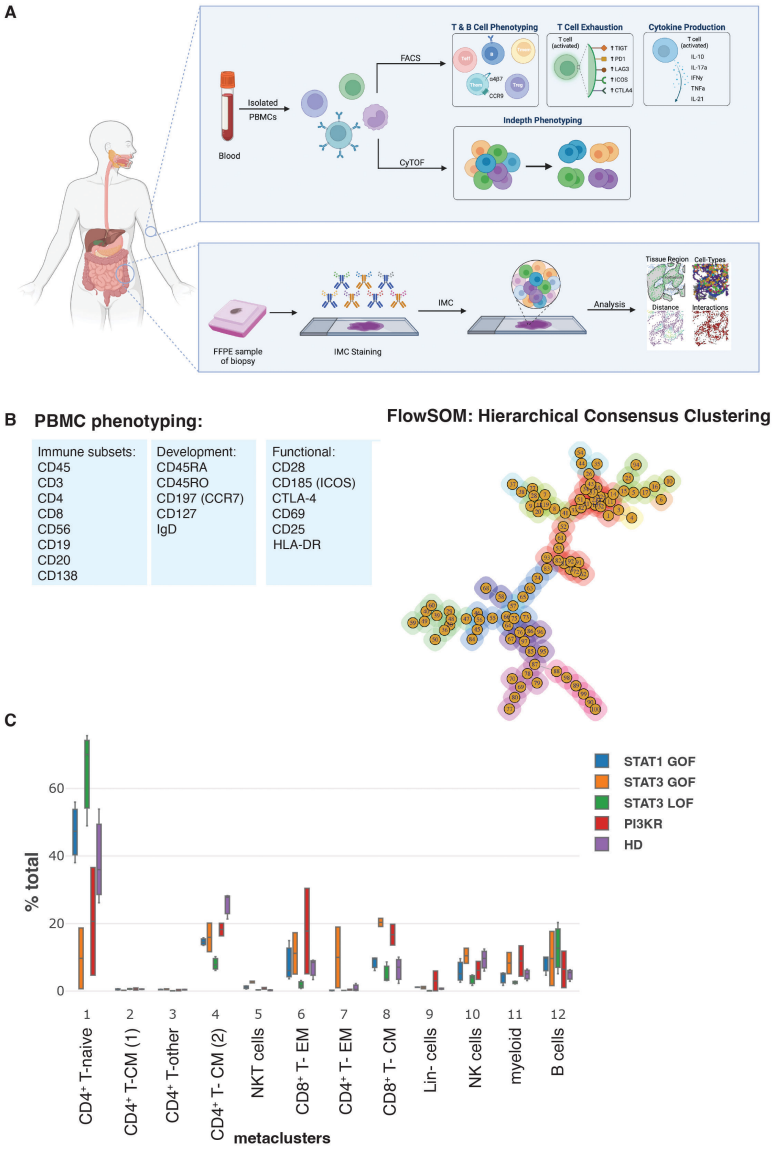
ID	Sex	IEI	Affected gene	Variant	Class	CyTOF	FACS	IMC	IST before IMC sample?
P1	F	STAT3-HIES	STAT3	p.V637M	LP	Yes	Yes	No	NA
P2	F	APDS2	PI3KR1	c.1425+2T>A	P	No	Yes	Yes	IVIG
P3	F	STAT3-HIES	STAT3	p.H410Y	LP	Yes	Yes	No	NA
P4	M	APDS2	PI3KR1	c.1425+1G>A	P	Yes	Yes	Yes	IVIG
P5	M	APDS1	PI3KCD	p.E1021K	P	No	Yes	No	NA
P6	F	STAT3-HIES	STAT3	p.V637M	LP	No	Yes	No	NA
P7	F	STAT1GOF	STAT1	p.R274Q	P	Yes	Yes	No	NA
P8	F	STAT1GOF	STAT1	p.K388E	LP	Yes	Yes	No	NA
P9	F	STAT1GOF	STAT1	p.R274Q	P	Yes	Yes	No	NA
P10	?	STAT3GOF	STAT3	NA	LP	Yes	Yes	No	NA
P11	?	STAT3GOF	STAT3	NA	LP	Yes	Yes	No	NA
P12	F	STAT3GOF	STAT3	p.P471R	LP	No	No	Yes	Anti-IL6, anti-α4β7, fludarabine
P13	F	STAT1GOF	STAT1	p.V266I	VUS	No	No	Yes	No

IMC = Imaging Mass Cytometry, IST = immunosuppressive therapy, IVIG = intravenous immunoglobulins, LP = likely pathogenic, P = pathogenic,

PIRD subgroups display differential naïve and memory T cell distributions and can be distinguished based on T cell homing, cytokine production and activation/exhaustion status

First, we performed unsupervised clustering of extracellular markers investigated with CyTOF from peripheral blood of 10/13 patients and three healthy controls (Figure 1B). Clusters allowed the distinction of multiple T and B cell subsets, myeloid cells and NK cells (Figure 1C), and showed variation between the different monogenetic PIRD patients. Specifically, all patients showed lower proportions of CD4+ central memory T cells in blood compared to healthy donors, which was particularly low in STAT3-HIES (STAT3-LOF). Naive CD4+ T cell proportions were variable, but seemed low in STAT3-GOF , and APDS (PI3KR), and STAT3-GOF and APDS patients had higher proportions of CD4+ effector memory, as well as CD8+ central memory T cells compared to healthy donors. CD8+ effector memory T cells were reduced in STAT3-HIES. STAT3-HIES also showed low proportions of NK cells and myeloid cells compared to the healthy donors and other PIRD subgroups. Together, variation in the peripheral immune compartment seemed to be largely driven by differences in the naive and memory CD4+ and CD8+ T cell populations.

Figure 1: CD4+ naïve and central memory T cells and CD8+ central memory T cells show variation between groups. (A) *Overview of the CyTOF, FACS and IMC workflow. Blood was drawn from patients with pathogenic or likely pathogenic variants that caused STAT1GOF, STAT3-HIES, APDS or STAT3GOF. Seven patients suffered from gastrointestinal symptoms for which they underwent a gastro/endoscopy. The PBMCs were isolated and used in FACS and CyTOF analysis. Tissue biopsies were stored in FFPE. Sections were placed on a glass slide and stained with metal labeled antibodies. Regions are ablated and single-cell data is analyzed for cell-types, interactions and distance.*
(B) CyTOF was performed on 10/13 patients and 3 healthy controls. Extracellular markers were analyzed using FlowSOM's hierarchical consensus clustering. (C) CD4+ CD45RA+ and CD4+CD27+CD45RO+ and CD8+CD27+CD45RO+ could distinguish between PIRD subgroups and healthy controls.



Flow cytometry data confirmed this variation: Here we also found significant variation within the CD4+CD27+/-CD45RO+ and CD8+CD27+/-CD45RO+ populations of the different groups (p=0.03 and p=0.04), and the same differences between PIRD subgroups as described previously, although they were not statistically significant.

Next, in-depth functional and phenotypical classification of T cells was performed using panels for intracellular cytokine production, T_{reg} and inhibitory receptors, T cell homing receptors, T cell activation and exhaustion, B cell subsets and molecules involved in B cell activation for in-depth FACS analysis in 11/12 patients and ten healthy controls (Figure 2A). First, we performed conventional gating of all the subsets and calculated the relative frequency of all the populations and extracted the mean fluorescence intensity of the inhibitory receptors. To determine differential populations between groups, we selected populations of cells by manual gating (**Supplementary Figure 2)** and extracted 68 populations. We then prepared the data for a PCA with oblique rotation. There were too few samples to explain 68 populations, expressed by the Kaiser-Meyer-Olkin (KMO) measure of common variance. The 15 populations with the poorest common variables were therefore excluded resulting in acceptable PCA prerequisites, with a KMO of 0.65 and a highly significant Bartlett's test. Three principal components, containing 42 populations, were able to explain 65% of the variance within the dataset. Together, these three components enabled us to distinguish between the PIRD subgroups and healthy controls (Figure 2B).

We then performed univariate analysis on these 42 populations and found that 26 populations were significantly different between the five groups when corrected for multiple testing (**Supplementary Figure 3**). These consisted predominantly of populations determined by cytokine expression, T cell homing and T cell exhaustion. We found that STAT1GOF and STAT3-HIES were hallmarked by reduced CD8+ effector/memory populations, reduced skin and gut primed T cells, increased PD1 expressing CD8+ T cells and increased naive B cells. STAT1GOF T_{reg} cells showed high ICOS expression, while STAT3-HIES $CD21^{low}$ B cells showed reduced BAFF receptor expression. APDS and STAT3GOF showed exhausted CD4+ T cells that were LAG3+PD1+ and less capable of IFN-γ production (Figure 3A-B). They showed more activated CD8+ T cells that were LAG3+PD1+ but still capable of GzmB production (Figure 3C). Moreover, they showed increased $CD21^{low}$ B cells with reduced BAFF receptor expression (Figure 3D) and STAT3-GOF showed more IL-17a+ T cells, increased CD27-IgD- B cells, cytokine producing T_{reg} cells, increased skin

and gut homing (Figure 3E) and reduced naive B cells. Together, these populations can be used to distinguish between APDS, STAT1GOF, STAT3GOF and STAT3-HIES.

Figure 2: In-depth phenotyping could separate patients from healthy controls and could distinguish between IEI types. *A) FACS analysis was performed on the PBMCs of 11 patients and 10 healthy controls using six panels related to T cell function and B cell subsets (created with BioRender). B) The relative abundance of 68 different T, B, NK and monocyte cell populations was extracted and PCA was performed, which revealed that 42 variables formed three clusters that could distinguish between groups.*

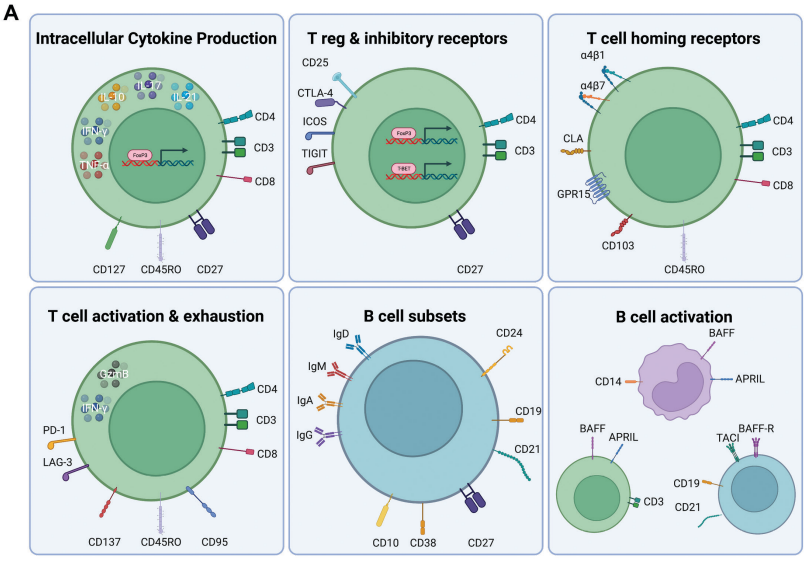
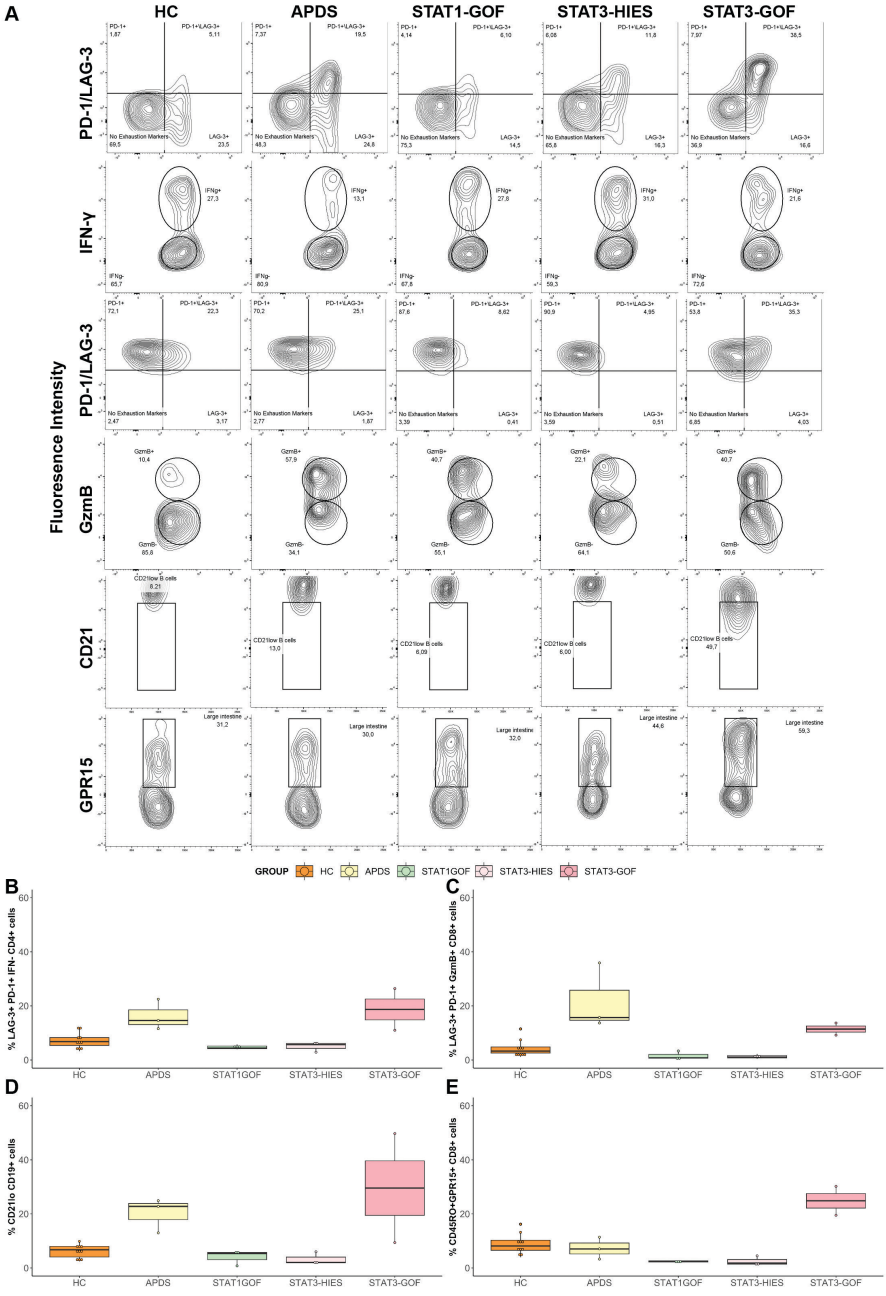


Figure 3: Exhausted CD4+ and activated CD8+ T cells, CD21^{lo} B cells and colon homed CD8+ T cells differed across the IEI groups. *A) Examples of gating strategies for exhausted (PD-1+LAG3+IFN-γ-CD4+ T cells, activated (PD-1+LAG3+IFN-γ+) CD8+ T cells, CD21^{lo} (CD21$^{dim/-}$CD19+) B cells and colon (GPR15+CD45RO+) CD8+ T cells. B-E all subsets show significant variation across the IEI groups and healthy controls.*



Inflamed intestinal tissue of PIRD patients displays limited epithelial T cell infiltration and reduced T cell differentiation and activation.

Finally, we investigated whether the distribution of cell subsets in the peripheral blood reflected that of T cells in the inflammatory microenvironment. To this aim, we performed IMC on 13 inflamed tissue biopsies of two APDS, one STAT1GOF and one STAT3GOF patients and compared it to 13 uninflamed tissue biopsies of 5 controls. First we performed hierarchical clustering analysis of immunomarker expression within the intestinal epithelium and stroma (Figure 4).

This showed that PIRD patients immunomarker expression within the epithelium clustered separately from that of uninflamed controls. PIRD patients could be distinguished by reduced expression of basal immune markers (CD3, CD4 and CD20), reduced expression of T cell lineage markers (FOXP3, Tbet, GATA3), reduced expression of PD1 and reduced cytokine expression (IFN-γ, IL-17A). Clustering of all epithelial and stromal cells and clustering of unannoted clusters are shown in Supplementary Figure 4 and 5.

Additionally, univariate analysis of the epithelium (Figure 5A-C) showed a similar CD45 expression, suggesting a similar degree of immune infiltration in the inflammatory microenvironment. Despite this, we found significantly lower CD3, CD4 and CD20 expression in all PIRD patients. APDS patients showed reduced CD45RO expression, suggesting reduced T effector/memory populations. Moreover, APDS and STAT3GOF patients showed reduced FOXP3 expression, suggesting reduced T_{reg} populations. Other markers for differentiated T cells, like Tbet (T helper 1) and GATA3 (T helper 2), were reduced in all PIRD patients Finally, all PIRD patients showed limited to absent ICOS and PD1 expression and IFN-γ production. Additionally, APDS and STAT3GOF patients also showed reduced IL-17 production.

Conversely, APDS patients showed more stromal immune infiltration and the STAT3GOF patient showed fewer stromal immune infiltration. CD3, CD8, CD45 and CD45RO expression was also increased in the stroma of APDS patient (Figure 5D-F), while stromal T cell infiltration was similar to controls in STAT1GOF and reduced in STAT3GOF patients. The increased T cell stromal T cell infiltration in APDS is likely caused by epithelial to stromal shift of CD8+ T cells, while the decreased T cell infiltration of STAT3GOF is likely caused by an absence of CD4+ T cells. We also found increased T effector/memory populations in the APDS and STAT1GOF patients. Like the epithelial T cells, stromal T showed reduced expression of PD1 and IFN-γ production in all PIRD patients. Co-localization of CD3, CD4, CD8,

CD45ORO and IFN-γ further confirmed these marked differences of infiltration of T cells in the inflammatory microenvironment (Figure 5G).

Figure 4: Immune subsets, T cell lineage markers, activation markers and cytokines in the intestinal epithelium can distinguish between PIRD and non-IEI patients. *A) Ilastik was used for extraction of single cell data from 13 biopsies of 4 PIRD patients and 13 uninflamed biopsies of 4 patients with Crohn's disease in remission. B) Hierarchical clustering of immune subsets, T cell lineage markers, activation markers and cytokines of epithelial regions could distinguish the 4 PIRD patients from the patients with Crohn's disease. C) Hierarchical clustering of the same markers in stromal regions could not distinguish between PIRD patients and Crohn's disease patients.*

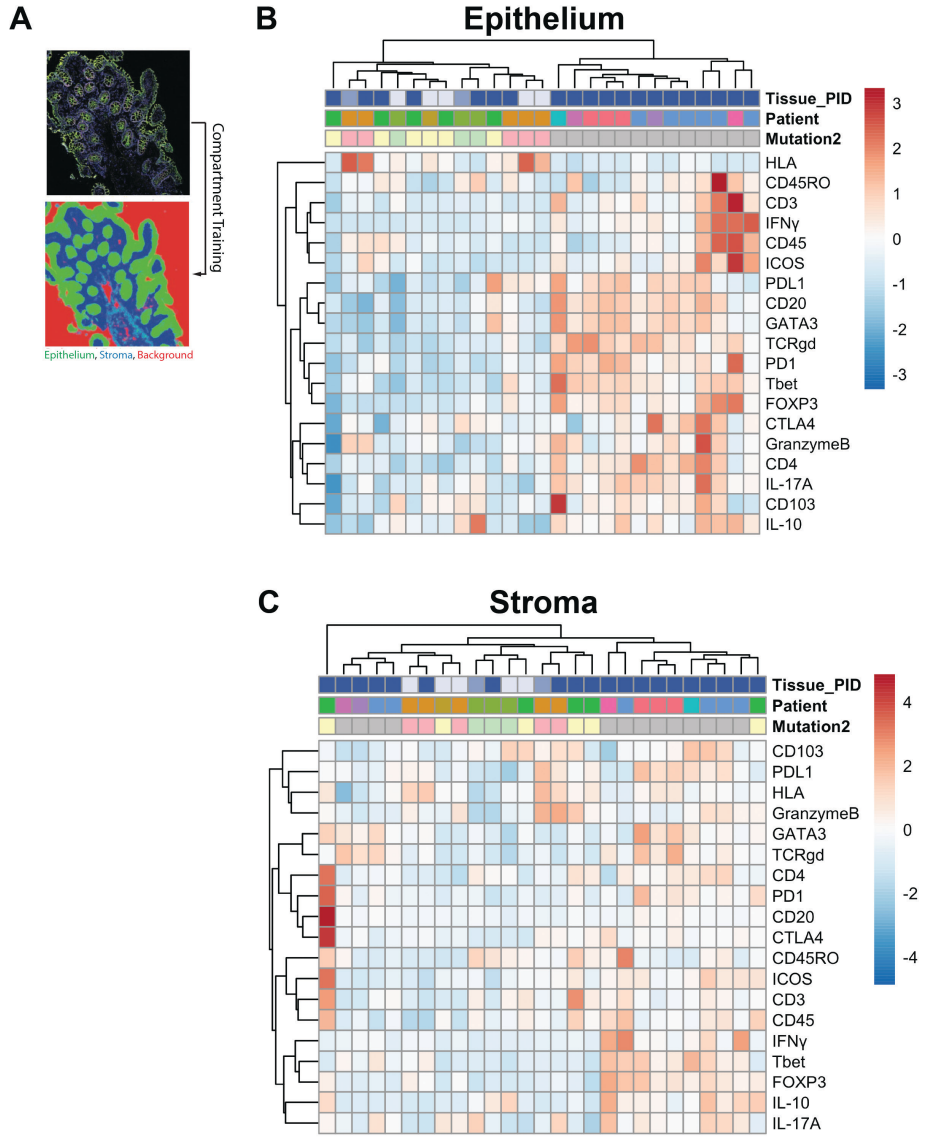
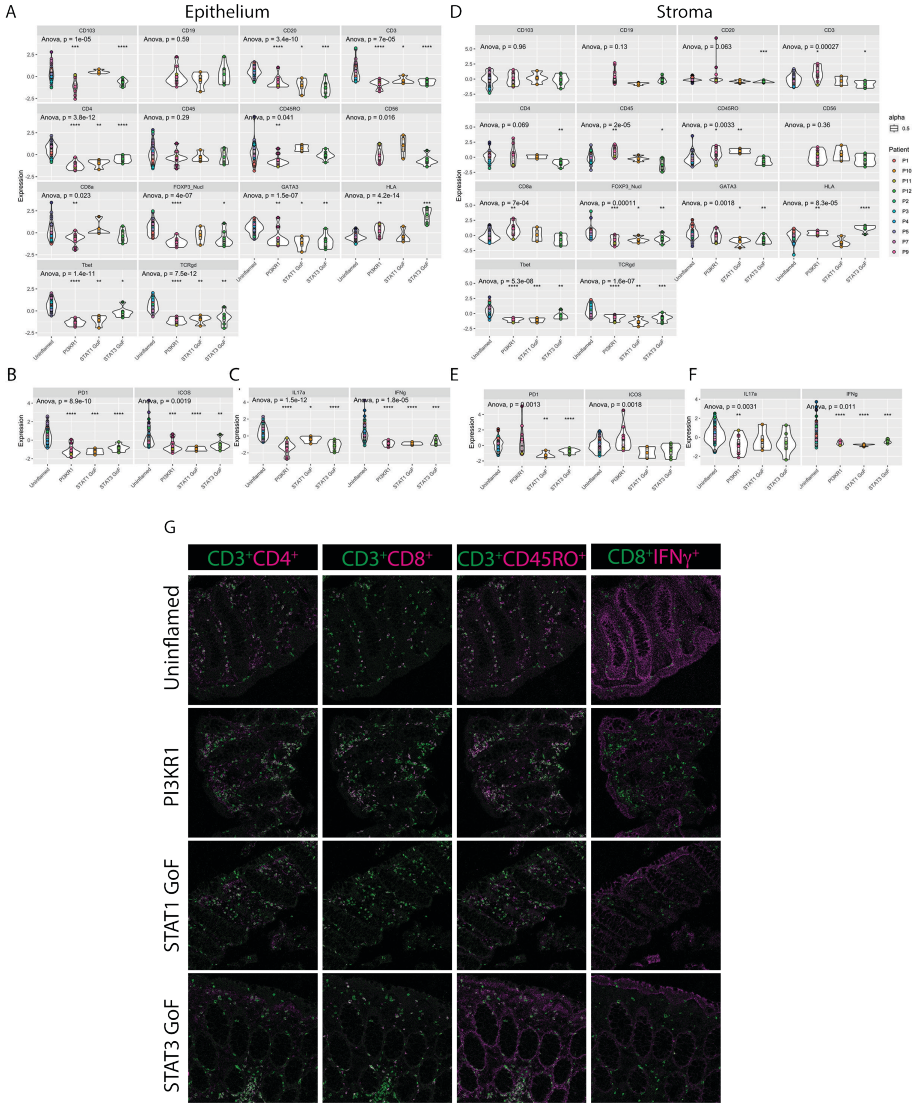


Figure 5: PIRD patients show fewer epithelial T cells and less epithelial and stromal T cell activation.
Univariate analysis (ANOVA) of the epithelium markers (A-C), showed lower CD3 and CD4 expression in all PIRD patients, normal CD19 expression, but lower CD20 expression, reduced ICOS and PD1 expression and IFN-γ production. APDS patients showed reduced CD45RO expression. APDS and STAT3GOF reduced FoxP3 expression and reduced IL-17 production.
In the stroma (D-F) APDS patients showed increased CD3, CD8, CD45 and CD45RO expression and reduced IL-17 production. The STAT1GOF patient showed increased CD45RO expression and the STAT3GOF showed reduced CD3 and CD4 expression. All PIRD patients showed reduced PD1 expression and IFN-γ production. (G) PIRD patients show decreased expression of IFN-γ, despite a varying presence of intestinal T cells.
**p<0.05, **p<0.01, ***p<0.001*



In conclusion, intestinal immunomarker expression showed little overlap with immunomarkers found in the blood. Instead, immune infiltrates expressed less T cell markers and potentially showed little signs of T cell activation.

DISCUSSION

This is the first study that directly compares the peripheral immune phenotype of different types of PIRD and explores the intestinal phenotype. Peripheral T cells subsets could distinguish between PIRD subgroups. Conversely, intestinal T cells showed signs of limited infiltration, activation and maturation.

Involvement of the T cell compartment has previously been described for all PIRD that were studied.[14,16–25] This study confirms data from those previous reports, such as an increased transitional B cells and reduced class switched memory B cells in APDS [17,23], decreased CD4 T cell IL-17 production in STAT1GOF and reduced effector/memory T cell fractions and class-switched memory B cells in STAT1GOF and STAT3-HIES [24,27–29,31,32], and reduced naive CD4+ T cells in STAT3GOF. [20,21,45] However, we did not observe previously reported lymphopenia or reduced naïve T cell fractions in APDS [17,2317,23] , a relative increase of IL-17 production by T_{reg} and CD8+ effector/memory cells in STAT3-HIES [24,28,31] , or normal levels of switched-memory B cell fractions and cytokine production in T_{reg} populations of STAT3GOF patients. [20,21,43] This further demonstrates that the immune phenotype of PIRD subgroups can be highly variable, as has previously been described. [14,16–25] It also confirms the overlap in the immunophenotype of STAT1GOF and STAT3-HIES patients that has previously been shown. [24,27–29,31,32]

The profound differences within the peripheral T cell compartment did not reflect the differences within the intestinal T cell compartment, where we observed reduced infiltration of T cells and reduced expression of T cell activation markers in the epithelium compared to uninflamed tissue of patients with Crohn's disease. Additionally, while peripheral T cells of STAT3GOF patients showed higher levels of gut homing markers, these were reduced in the intestinal tissue of one STAT3GOF patient. Moreover, while peripheral CD45RO+ T cells of STAT1GOF were reduced, we found increased intestinal CD45RO expression in one STAT1GOF patient. This lack of T cell involvement contradicts earlier research in PAD patients without monogenetic disease, where T cell enriched lymphocytic infiltrates have been shown. [34,37] Moreover, this finding also indicates that the inflammatory

microenvironment of enteropathy in PIRD might differ from that of IBD, where earlier research has shown increased infiltration of activated CD8+ T cells.[46]

These findings could potentially be explained by the dysfunctional peripheral T cell compartment found in PIRD patients. Alternatively, sampling bias may have been introduced, since IMC analyses include only small amounts of tissue and tissue sampling peripheral blood sampling dates and patients were not matched. However, multiple samples from multiple tissue sites were included and showed similar findings. Moreover, multiple samples from different dates were used for one APDS patient and the STAT3GOF patient and also showed similar findings. Even when we limit our conclusions only to the APDS patients, for whom paired samples were included, we did not find an overlap in peripheral phenotype and the intestinal inflammatory microenvironment. Immunosuppression usage could be an additional explanation for this discrepancy in T cell phenotype, however this was only the case in the STAT3GOF patient. In conclusion, this study shows that the role of T cells in the inflammatory microenvironment in PIRD associated enteropathy is potentially limited.

Important limitations need to be taken into consideration. First, this study included only a small number of patients and additionally multiple statistical tests have been performed to analyze the data, possibly resulting in type II errors. To account for this, we have adjusted the critical p value according to the Holms-Bonferroni method. This however cannot correct the inadequate sample size and this cohort certainly does not reflect the heterogeneity of the immune cell compartment of the PIRD that were studied here. Second, this cohort was sampled cross sectionally and we could therefore not correct for disease duration and medication use. Third, we used non-IEI patients as controls for the IMC data, possibly biasing our findings. Relative expression of immune markers of IEI patients compared to non-IEI patients could very well be reduced in general, and this finding might not be specific for the PIRD described here. Finally, we only analyzed relative expression of immune markers in the IMC data and did not perform in-depth analysis of immune marker co-expression, which could specifically reveal the intestinal immune subsets that are present.

We conclude that functional T cell markers can potentially distinguish between PIRD subgroups and between STAT1GOF and STAT3-HIES. This approach should be further investigated in a larger, prospectively sampled cohort of PIRD patients that includes other PIRD subgroups. Moreover, we have also shown that the intestinal

T cell compartment might not be as important in intestinal pathology in PIRD as previously thought. Future studies should therefore also focus on other intestinal immune subsets to further elucidate immune cells that play a vital role in intestinal pathology in PIRD.

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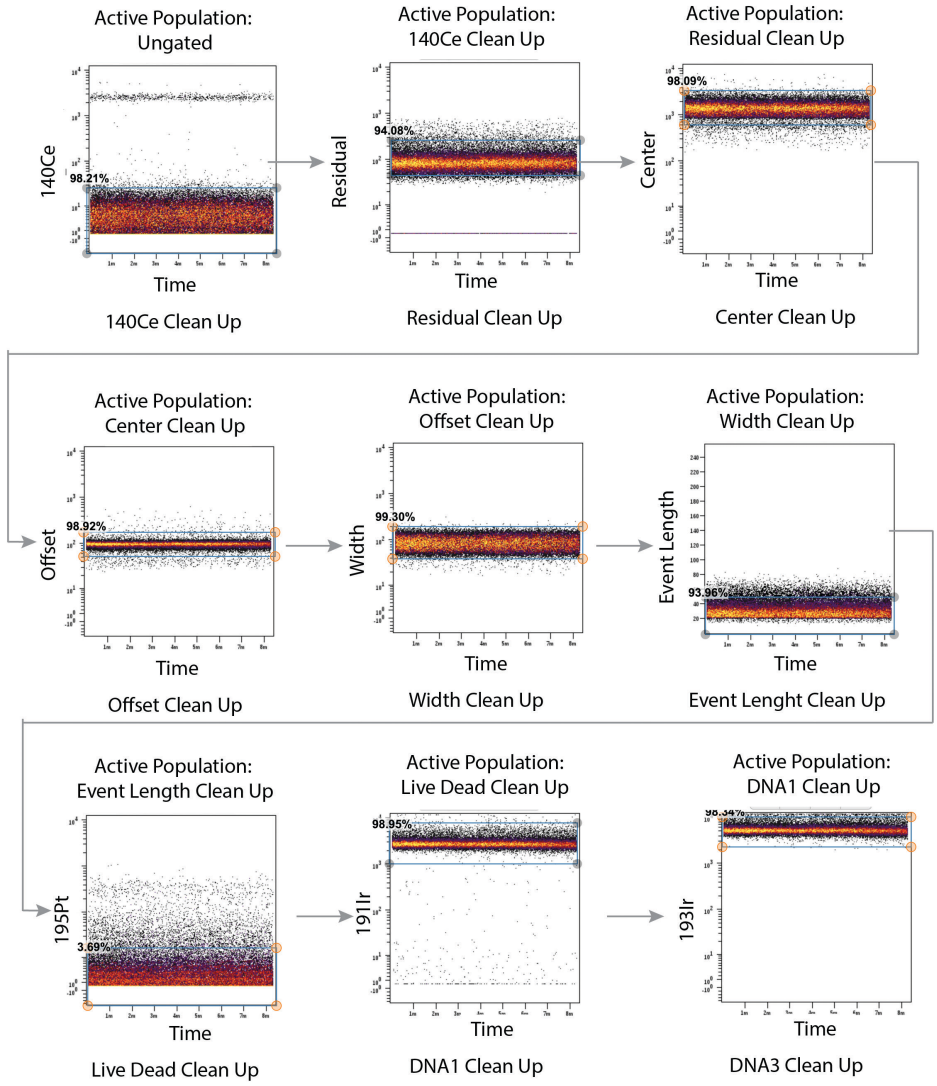
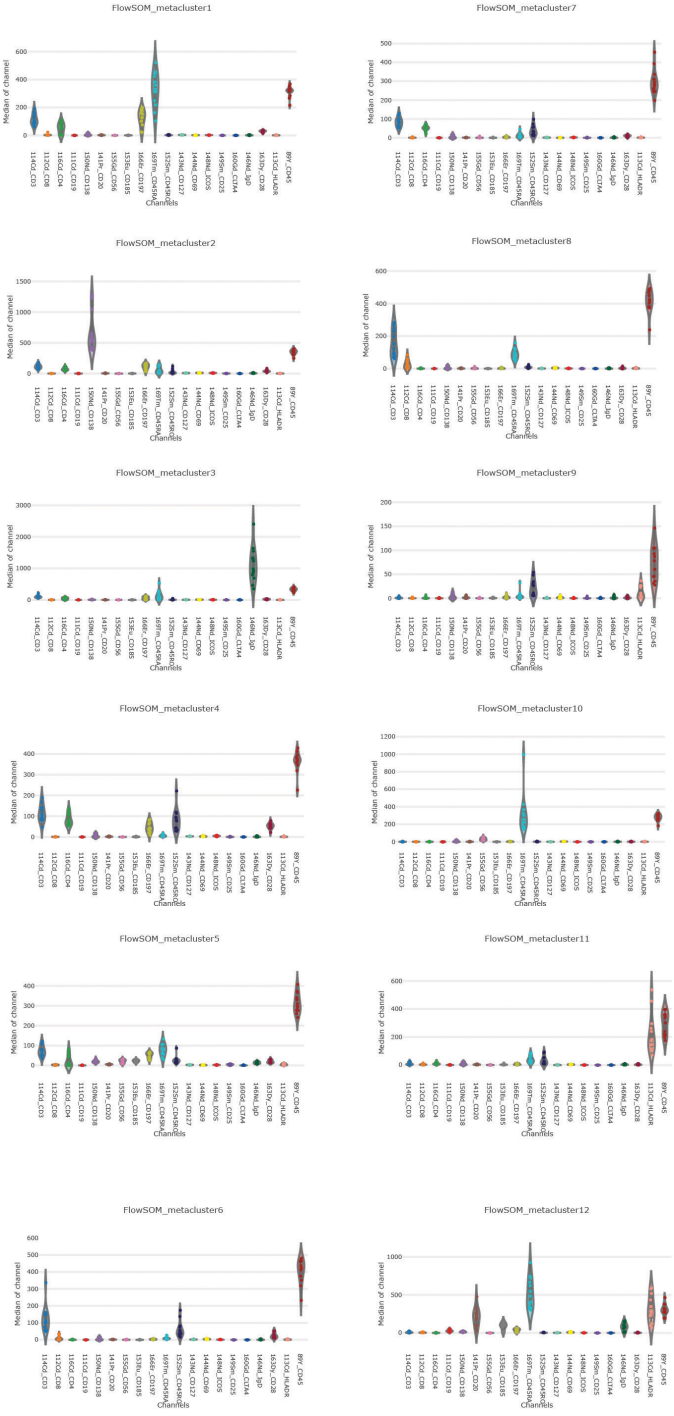
SUPPLEMENTARY FIGURES

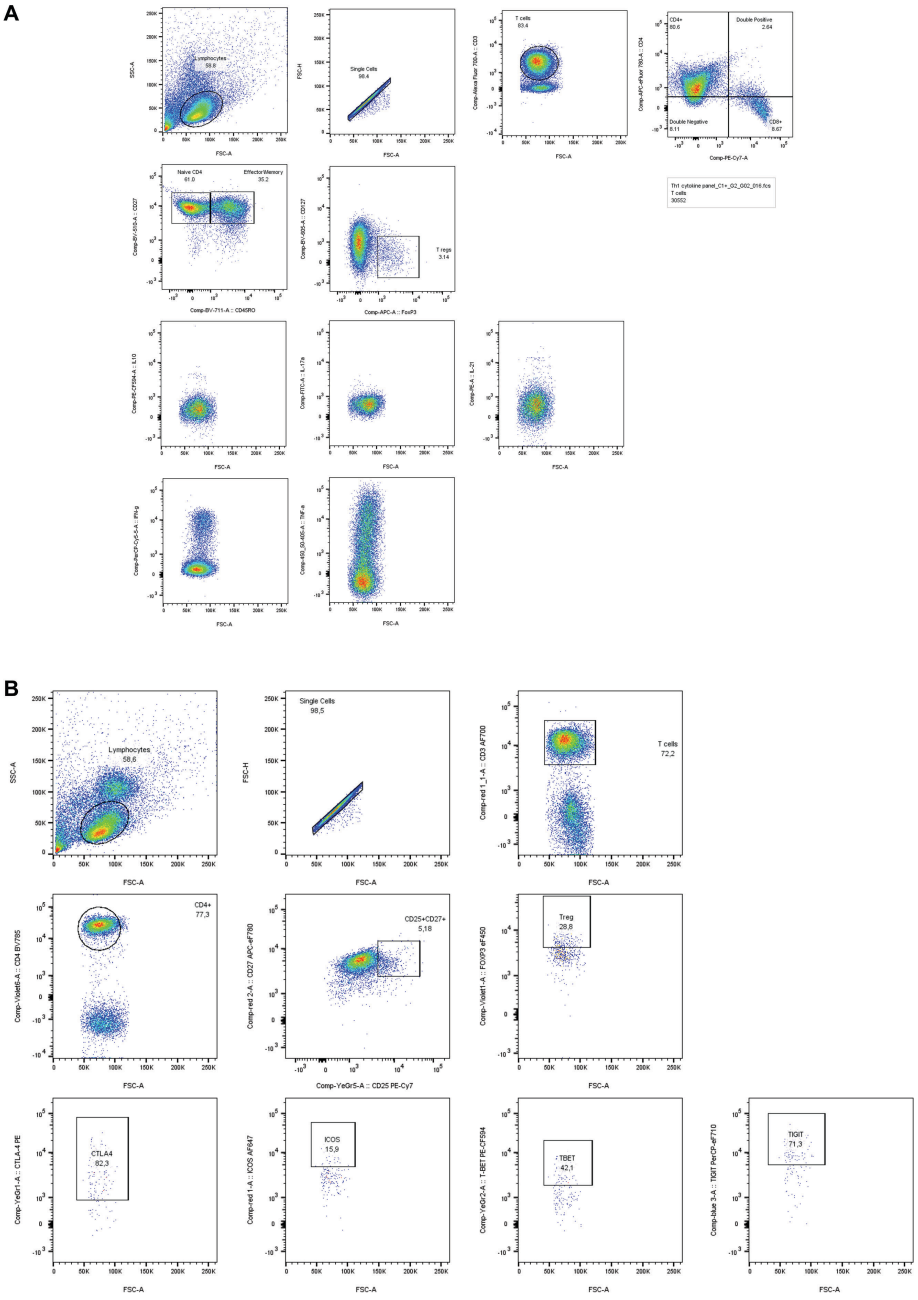
Supplementary Figure 1: Representative gating of CyTOF data demonstrating data clean up and live gate.



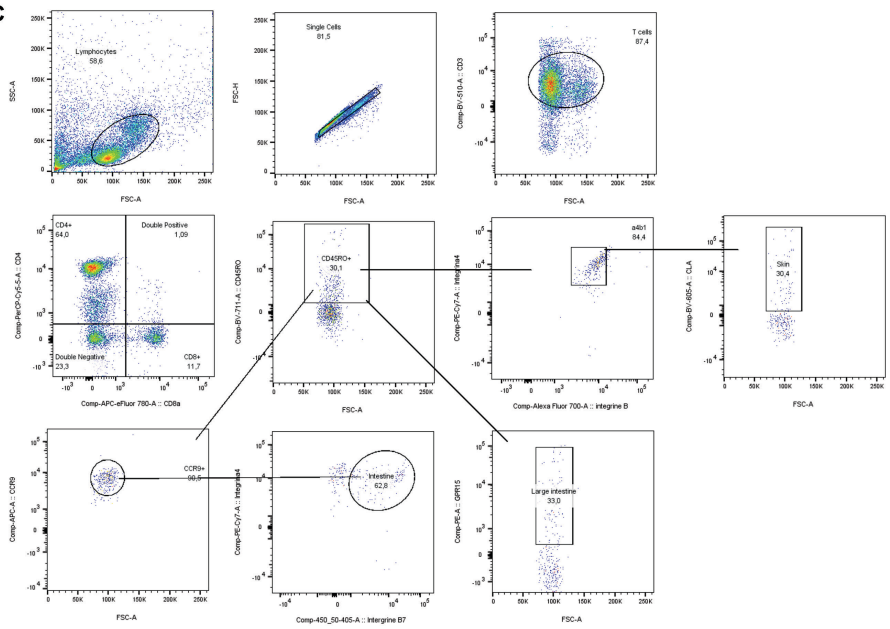
Supplementary Figure 2: Metaclusters generated by FlowSOM



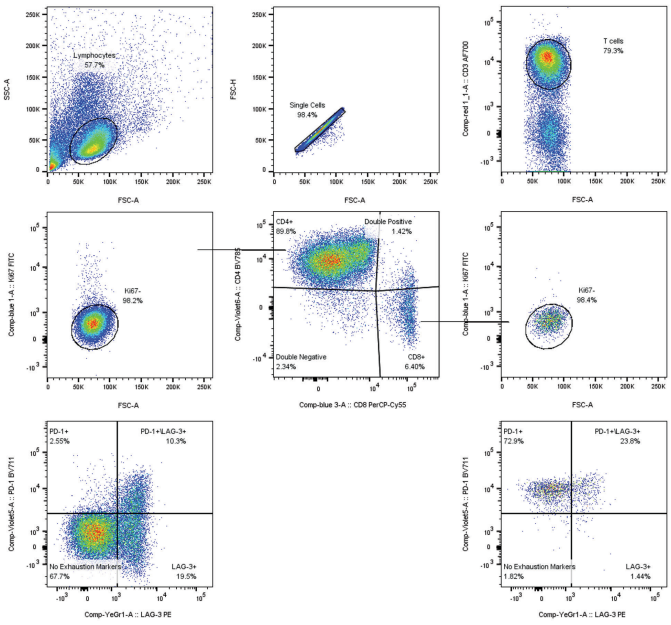
Supplementary Figure 3: Representative gating of FACS data for the six panels. *A) Cytokine Panel, B) T_{reg} Panel, C) Homing Panel, D) Exhaustion Panel, E) B cell Panel, F) B cell Activation Panel*

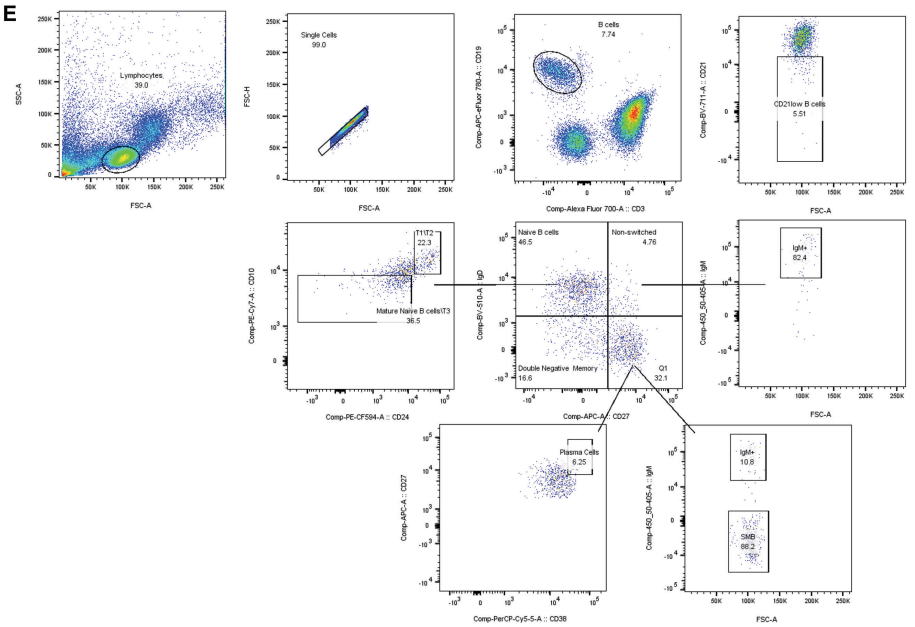


C

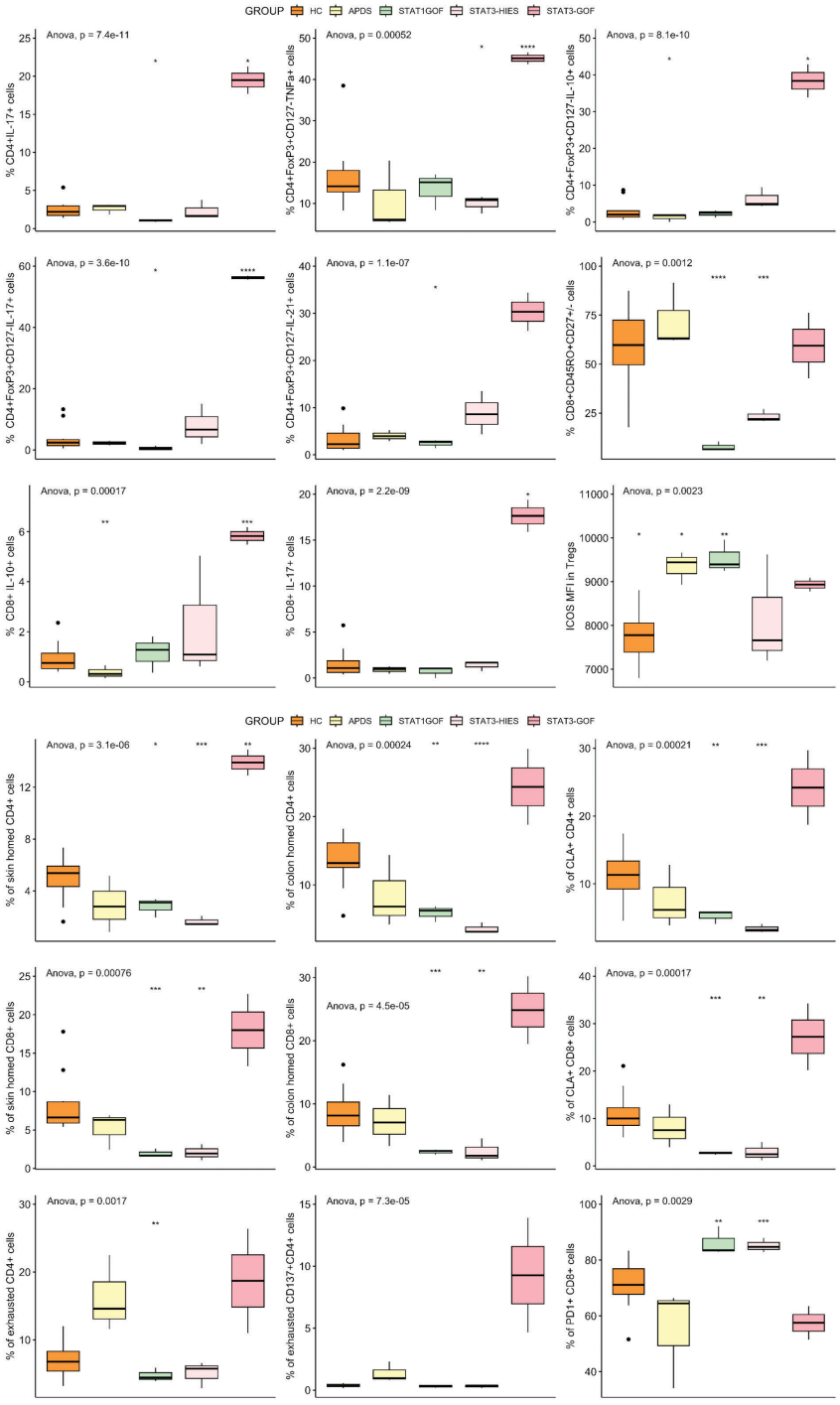

Lymphocytes
58,6
SSC-A
FSC-A
Single Cells
81,5
FSC-H
T cells
87,4
Comp-BV-510-A :: CD3
CD4+
64,0
Double Positive
1,09
Double Negative
23,3
CD8+
11,7
Comp-PerCP-Cy5-5-A :: CD4
Comp-APC-eFluor 780-A :: CD8a
CD45RO+
30,1
Comp-BV-711-A :: CD45RO
a4b1
84,4
Comp-PE-Cy7-A :: Integrin4
Comp-Alexa Fluor 700-A :: integrine B
Skin
30,4
Comp-BV-605-A :: CLA
CCR9+
99,5
Comp-APC-A :: CCR9
Intestine
62,8
Comp-PE-Cy7-A :: Integrin4
Comp-450_50-405-A :: Integrine B7
Large intestine
33,0
Comp-PE-A :: GPR15

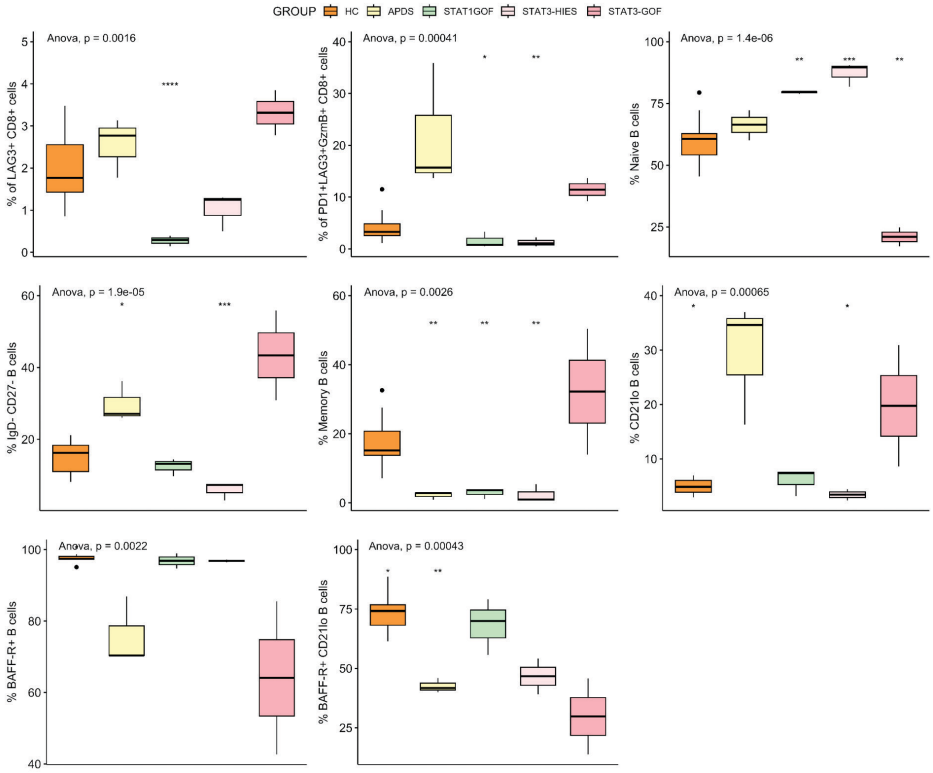
D


Lymphocytes
57.7%
SSC-A
FSC-A
Single Cells
98.4%
FSC-H
T cells
79.3%
Comp-red L_1-A :: CD3 AF700
Ki67-
98.2%
Comp-blue 1-A :: Ki67 FITC
CD4+
89.8%
Double Positive
1.42%
Double Negative
2.34%
CD8+
6.40%
Comp-Violet5-A :: CD4 BV785
Comp-blue 3-A :: CD8 PerCP-Cy55
Ki67-
98.4%
PD-1+
2.55%
PD-1+LAG-3+
10.3%
No Exhaustion Markers
67.7%
LAG-3+
19.5%
Comp-Violet5-A :: PD-1 BV711
Comp-YeGr1-A :: LAG-3 PE
PD-1+
72.9%
PD-1+LAG-3+
23.8%
No Exhaustion Markers
1.82%
LAG-3+
1.44%

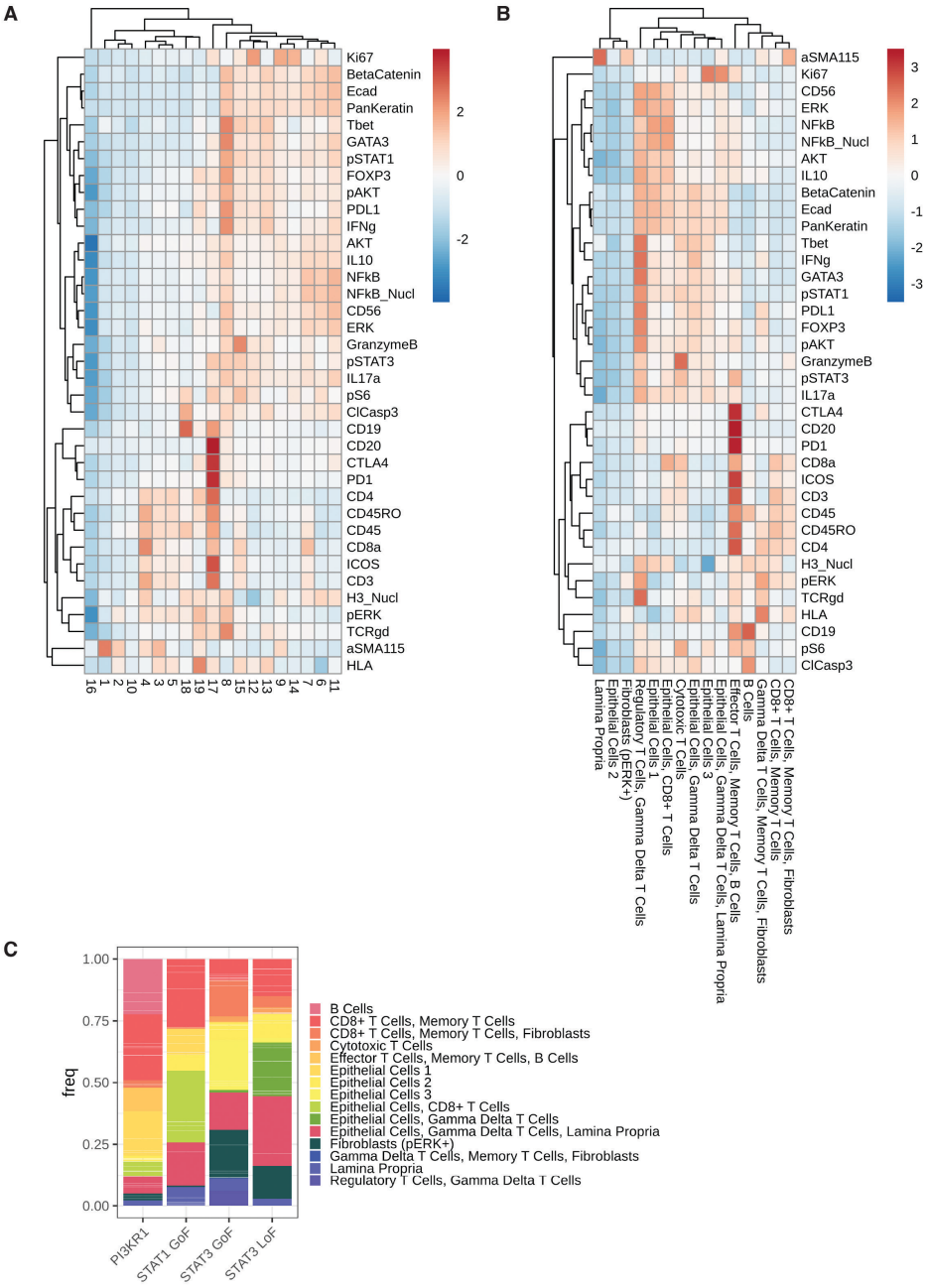

E
Lymphocytes
39.0
SSC-A
FSC-A
Single Cells
99.0
FSC-H
B cells
7.74
Comp-APC-eFluor 780-A :: CD19
Comp-Alexa Fluor 700-A :: CD3
CD21low B cells
5.51
Comp-BV-711-A :: CD21
T1/T2
22.3
Mature Naive B cells/T3
36.5
Comp-PE-Cy7-A :: CD10
Comp-PE-CF594-A :: CD24
Naive B cells
46.5
Non-switched
4.76
Double Negative Memory
16.6
Q1
32.1
Comp-BV-510-A :: IgD
Comp-APC-A :: CD27
IgM+
82.4
Comp-450_50-405-A :: IgM
Plasma Cells
6.25
Comp-APC-A :: CD27
Comp-PerCP-Cy5-5-A :: CD38
IgM+
10.8
SMB
88.2
Comp-450_50-405-A :: IgM
F
PBMC's
52.5
SSC-A
FSC-A
Single Cells
99.6
FSC-H
B cells
14.0
CD3-CD19-
8.16
T cells
75.6
Comp-BV-711-A :: CD19
Comp-Alexa Fluor 700-A :: CD3
Monocytes
34.6
NK cells
40.0
Comp-BV-510-A :: CD14
Comp-PE-Cy7-A :: CD56
BAFF+
5.12
Comp-FITC-A :: BAFF
BAFF-R+
13.8
Comp-PerCP-Cy5-5-A :: BAFF-R
TACI+
6.38
Comp-APC-A :: TACI
APRIL+
15.1
Comp-PE-A :: APRIL

Supplementary Figure 4: Populations showing significant variation between PIRD groups.

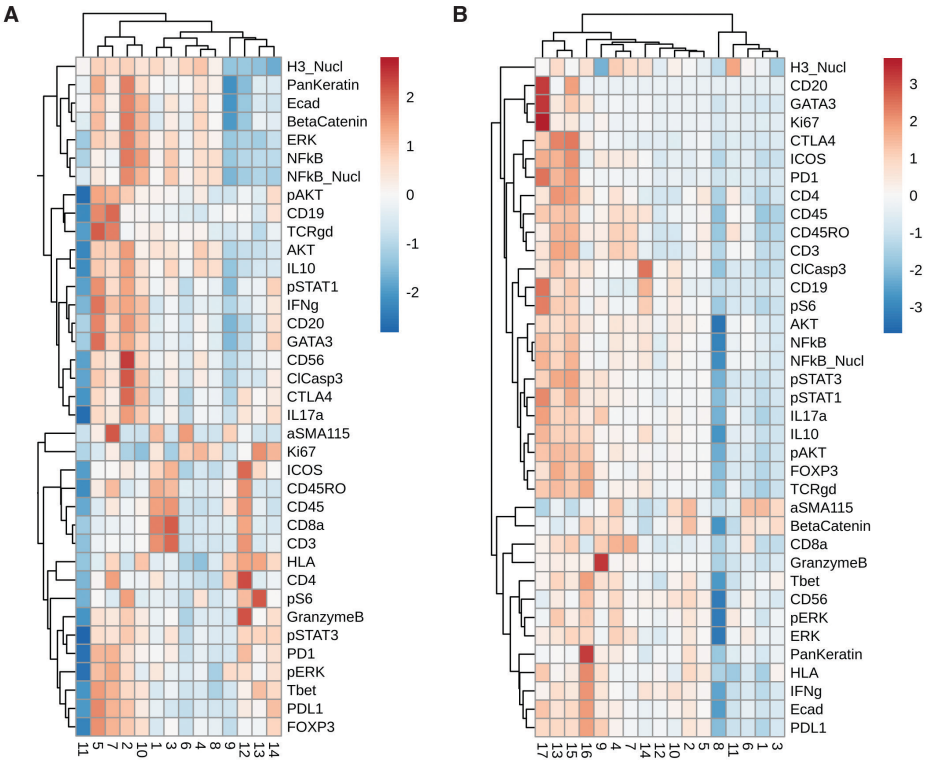



GROUP HC APDS STAT1GOF STAT3-HIES STAT3-GOF
Anova, p = 0.0016
% of LAG3+ CD8+ cells
Anova, p = 0.00041
% of PD1+LAG3+GzmB+ CD8+ cells
Anova, p = 1.4e-06
% Naive B cells
Anova, p = 1.9e-05
% IgD- CD27- B cells
Anova, p = 0.0026
% Memory B cells
Anova, p = 0.00065
% CD21lo B cells
Anova, p = 0.0022
% BAFF-R+ B cells
Anova, p = 0.00043
% BAFF-R+ CD21lo B cells

Supplementary Figure 5: Clustering of all the cells localized in the epithelium and stoma.



Supplementary Figure 6: Unannoted Clusters of the Epithelium and Stroma.



CHAPTER 5

Feasibility of an *in vitro* T cell proliferation assay for predicting treatment response in complex common variable immunodeficiency disorder with non-infectious complications

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ABSTRACT

Background: Non-infectious complications resulting from immune dysregulation are the most important cause of morbidity and mortality in patients with common variable immunodeficiency disorder (CVID). Dysregulated T cells have been implicated as an important factor in the pathophysiology in CVID with non-infectious complications (also referred to as complex CVID, $CVID_c$). Due to the rarity of CVID and $CVID_c$, treatment guidelines and clinical trials investigating the efficacy of new immunosuppressants are lacking.

Objective: This study aimed to assess the feasibility of an *in vitro* T cell proliferation assay to predict individual treatment responses in $CVID_c$.

Methods: Peripheral blood mononuclear cells (PBMC) obtained from $CVID_c$ patients, CVID patients without complications ($CVID_i$) and healthy controls. They were stimulated using plate bound anti-CD3 and exposed to three concentrations of prednisone, azathioprine, sirolimus, ruxolitinib and leniolisib. Intracellular cytokine production was measured after two days and T cell proliferation after four days.

Results: Stimulation with anti-CD3 induced proliferation in $CD4^+$ and $CD8^+$ central memory and effector memory T cells in healthy controls, $CVID_i$ and $CVID_c$ ($p<0.01$). Prednisone was less effective at inhibiting T cell proliferation in $CVID_c$ patients with gastro-intestinal disease and azathioprine was less effective at inhibiting T cell proliferation in $CVID_c$ patients with interstitial lung disease. Intracellular cytokine production was inhibited with immunosuppressants, with no statistically significant differences between medication groups. Finally, successful *in vitro* inhibition of T cell proliferation in three out of four T cell subsets showed to match with clinical response in 72% of cases.

Conclusion: *In vitro* inhibition of T cell subset proliferation shows promise as a predictor of clinical response to immunosuppressive therapy in $CVID_c$.

INTRODUCTION

The burden of disease in common variable immunodeficiency disorder (CVID) patients includes infections and non-infectious complications. Non-infectious complications are the most important cause of morbidity and mortality in patients with CVID and occur in 30% - 60% of patients.[1,2] These non-infectious complications include lymphoproliferation, malignancies, auto-immune and inflammatory manifestations, such as auto-immune cytopenias, arthritis, systemic lupus erythematosus, enteropathy and granulomatous or lymphocytic interstitial lung disease (GLILD).[2–4]. These complications can manifest in various organ systems, are often difficult to treat and frequently relapse and are therefore often referred to as 'complex CVID' (CVIDc).[2–5]

GLILD occurs in 10 - 20% of CVID patients and is hallmarked by pulmonary granulomatous and lymphocytic infiltrates that consist of B- and T- lymphocytes.[6] These infiltrates can cause inflammation with subsequent pulmonary tissue destruction and fibrosis, which causes cough and dyspnea on exertion, and can ultimately lead to pulmonary hypertension and pulmonary failure.[4,7,8] Furthermore, enteropathy, a term describing any gastrointestinal disease (GI) where infectious causes have been excluded, occurs in 20 - 30% of CVID patients. Enteropathy is associated with a heterogenous presentation of inflammation that can be both acute, inflammatory bowel disease-like (IBD-like), and chronic in nature.[2,3,9,10] Enteropathy is often associated with lymphocytic colitis hallmarked by unspecific inflammation, intraepithelial lymphocytosis and lymphoid aggregates in the lamina propria. [9,11,12] Additionally, intestinal infiltrates have been reported to be enriched in T cells and diminished in plasma cells.[9,11,12] These infiltrates cause inflammation, pain, bloating and severe diarrhea that can lead to protein-loss, malnutrition, and CVIDc associated enteropathy is associated with decreased survival.[3,11]

Currently, there are no specific treatment guidelines for GLILD and enteropathy in $CVID_c$. d also been used, however with limited success.[3] Anecdotal success with anti-TNF-α therapy and vedolizumab has been reported.[20–23]

Individual treatment responses to current immunosuppressive therapies are thus heterogenous in GLILD and enteropathy, probably reflecting the heterogeneity of the underlying pathology of these complications in CVID patients. However, earlier findings may support that remission of these complications can be achieved by specifically targeting T cell effector functions, [15,23,24] as several papers have shown

that patients with GLILD and CVID enteropathy have increased activated CD4+ and CD8+ effector/memory cells and reduced T_{reg} functionality.[25,26].

Recently, new types of anti-inflammatory drugs, such as JAK/STAT inhibitors, PI3K inhibitors and new cytokine inhibitors to target dysregulated inflammation, have been approved for other (non-CVID) types of auto-immune/inflammatory diseases.[27–29] However, these new drugs are not yet widely available for CVID patients since phase III/IV clinical trials supporting their effectivity in CVID are lacking. Since CVID is a rare disease, it is not feasible to test each of these new drugs in randomized clinical trials. Instead, an *in vitro* or assay that could predict individual response to immunosuppressive therapy would be more appropriate to account for the heterogeneity and rarity of CVID. Currently, *in vitro* models that adequately model immunosuppression in auto-immunity/inflammatory disease in general have rarely been described and to date, no such assay has focused on the non-infectious complications of CVID.[30,31]

Moreover, *in vivo* models that consistently reproduce a CVID phenotype that includes non-infectious complications such as GLILD or enteropathy are also lacking.[32]

In this study, we describe the development and feasibility of an *in vitro* assay that specifically monitors the suppression of effector T cell function in CVID associated GLILD and enteropathy. Patient's isolated peripheral blood mononuclear cells were used for this assay to mimic the interaction between monocytes and T cells. We hypothesize that the efficacy of immunosuppressants on peripheral T cells is a surrogate marker for the efficacy of T cells in the inflammatory microenvironment in the tissue and that individual response in this assay therefore correlates with clinical responses of the included patients.

METHODS

Ethics Statement

The Medical Ethical Committee of the Erasmus MC University Medical Center in Rotterdam, The Netherlands (METC: 2013–026) provided ethical approval for all patients in this study. The technical committee for biological sample collection of the University Medical Center in Utrecht (UMCU), The Netherlands (TCbio: F10P50), provided ethical approval for all healthy controls in this study. Written informed consent was obtained from all patients and controls according to the Declaration of Helsinki.

Study Population and Sample Collection

We recruited healthy volunteers working at the UMCU, who were not associated with this study, as healthy controls (HC). CVID patients with non-infectious complications and age-matched CVID patients without non-infectious complications were recruited for validation. Patients were diagnosed with CVID according to the European Society for Immunodeficiencies criteria and were included at the outpatient clinics of the UMCU.[33] CVID patients were eligible if they were seven years or older.

Sample Processing and drug exposure cultures

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation (GE Healthcare-Biosciences, AB), and frozen in liquid nitrogen until use. Cells were subsequently thawed, counted and plated at 100,000 live cells per condition in 96 wells plates containing plate bound anti-CD3 antibodies (αCD3, Biolegend). Cells were cultured in the presence of 0.1, 1 or 10 µmol of prednisone, azathioprine, sirolimus, ruxolitinib or leniolisib. Cells cultured without αCD3 and without immunosuppressants were used as negative control. Cells cultured with αCD3 without immunosuppressants were used as positive control and cells cultured with beads coated with anti-CD3/anti-CD28 antibodies were used as proliferation control.

Proliferation

Cells were stained with the Cell Trace Violet kit (Thermofisher), according the manufacturer's instructions. Then cells were counted and plated and cultured for 96 hours. Next, cells were incubated with surface antibodies (Supplementary Table 1) for 30 min at 4 °C and washed. Finally, cells were measured on the LSR Fortessa (BD Biosciences). Naive T cells were defined as CD3+CD4+/CD8+CD45RO-CCR7+, central memory (CM) T cells were defined as CD3+CD4+/CD8+CD45RO+ CCR7+ and effector memory (EM) T cells were defined as CD3+CD4+/CD8+CD45RO+ CCR7-. Gating strategies are shown in supplementary figure 1.

Intracellular cytokine production

Cells were cultured for 48 hours and then restimulated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA, MilliporeSigma) and 1 µg/mL ionomycin (MilliporeSigma) for 4 hours at 37 °C with addition of monensin (Golgistop, BD Biosciences, 1:1500). Next, cells were incubated with surface antibodies (Supplementary Table 1) for 30 min at 4 °C and washed. Cells were then permeabilized with fixation/permeabilization reagent (eBioscience) for 30 min at 4 °C, washed, and incubated with the intracellular antibodies (Supplementary Table 1). Finally, cells were analyzed on the LSR Fortessa (BD Biosciences). Naive

T cells were defined as CD3+CD4+/CD8+CD45RO-CD27+, effector/memory (EM) T cells were defined as CD3+CD4+/CD8+CD45RO+CD27-/+ and regulatory T cells (T_{reg}) were defined as CD3+CD4+FoxP3+CD127-. Gating strategies are shown in supplementary figure 2.

***In vitro* efficacy**

We compared the performance of a cut-off and a Δ as measure for effectivity. Cut-offs were calculated by using the median proliferation level of all drugs at a concentration of 1μM in a T cell subset in healthy controls. The Δ was calculated by subtracting the median proliferation levels at a concentration of 1μM in a T cell subset from the median proliferation at a concentration 0μM in a T cell subset. When an immunosuppressant managed to reduce the proliferation below the cut-off or the absolute reduction was more than the Δ in an individual subject, the immunosuppressant was deemed effective.

Clinical efficacy

Clinical efficacy of immunosuppressants was acquired from the electronic patients records. An immunosuppressive regimen was deemed successful if an improvement of previously reported symptoms, leading to relapse-free remission, was described by the clinician.

Analysis and Statistics

We used FlowJo to acquire mean fluorescence intensities (MFI) and percentages of positive cells from flow cytometric data. We used R studio 3.2.0 for statistical analysis. Paired data was compared using aligned rank transformation ANOVA and continuous data was compared using Kruskal-Wallis tests.[34] P values below 0.05 were considered statistically significant.

RESULTS

Study population

Healthy controls (HC, n=10), CVID patients with only infectious complications ($CVID_i$, n=10) and CVID patients with complex disease ($CVID_c$, n=10) were recruited from our cohort (Table 1). The median age of the healthy controls was 44 years and 30% were male.

Table 1: Patient demographics and clinical information.

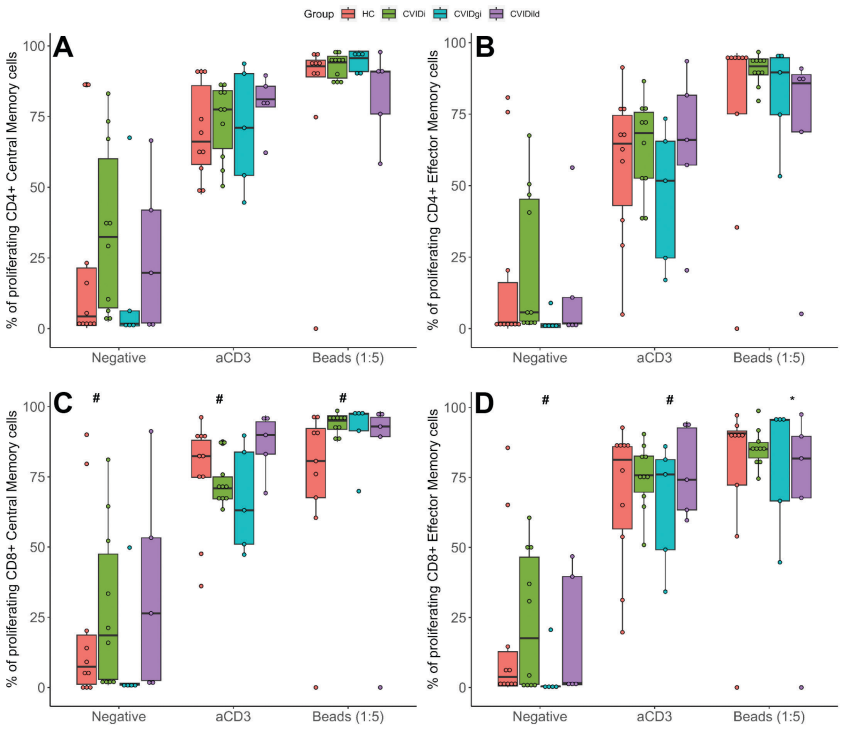
ID	Sample Date	Sex	Age	Gene?	IRT SD	IRT route	IC?	NIC (sample)	IT (sample)	IT (FU)
CVIDi1	23/03/2016	F	13	NA	0.398	SC	-	-	-	NA
CVIDi2	24/11/2016	M	13	-	0.223	SC	-	-	-	NA
CVIDi3	26/02/2020	M	16	-	0.469	IV	-	-	-	NA
CVIDi4	19/01/2017	M	25	NA	0.392	SC	-	-	-	NA
CVIDi5	15/02/2016	M	27	TACI	0.348	SC	-	-	-	NA
CVIDi6	31/10/2016	F	30	NA	0.611	IV	-	-	-	NA
CVIDi7	08/01/2016	M	37	NA	0.391	SC	-	-	-	NA
CVIDi8	20/03/2017	F	37	NA	0.631	IV	-	-	-	NA
CVIDi9	02/03/2016	F	47	NA	0.697	IV	-	-	-	NA
CVIDi10	10/02/2017	F	49	-	0.650	IV	+	-	-	NA
CVIDc1	11/04/2016	F	45	-	0.647	IV	-	ILD/GI	CsA	BS, RTX,
CVIDc2	17/03/2016	M	19	-	0.369	SC	-	ILD/GI	-	Pred, AZA, RTX+SIR
CVIDc3	02/10/2019	F	7	TACI	0.572	IV	-	ILD/GI	RTX+SIR	Pred, AZA, RTX+SIR
CVIDc4	20/04/2016	M	14	NA	0	-	-	GI	-	Pred, AZA, αTNF
CVIDc5	01/11/2021	F	48	-	0.656	IV	-	GI	-	-
CVIDc6	19/12/2016	M	16	TACI	0.293	SC	-	GI/AC	-	Pred, SIR
CVIDc7	03/06/2016	F	43	NA	0.476	IV	-	GI	αTNF	BS, AZA, αTNF
CVIDc8	05/09/2016	M	36	NA	0.421	IV	-	GI	-	BS, Pred, AZA
CVIDc9	28/06/2021	M	36	-	0.490	IV	-	ILD/GI/ SLE/AC	-	Pred, Pred+AZA
CVIDc10	14/03/2016	F	31	NA	0.533	IV	-	ILD	-	-

+ = Yes; - = No; AC = auto-immune cytopenias; AZA = azathioprine; BS = budesonide; CsA = cyclosporin A; $CVID_i$ = CVID with only infectious complications; $CVID_c$ = CVID with non-infectious complications; FU = during follow-up; GI = gastro intestinal complications; IC = infectious complication; ILD = interstitial lung disease; IRT = immunoglobulin replacement therapy; IT = immunosuppressive therapy; pred = prednisone; RTX = rituximab; sample = at the time of sampling; SD = standardized dose; sir = sirolimus.

Distribution of sex and age was similar for both CVID groups, although the median age was slightly higher in the $CVID_c$ group (33.5 vs 28.5 years). PID related genetic variants, standardized IRT dose and IRT injection route did not differ between the two groups. One $CVID_i$ patient suffered from severe bronchitis within three months before sampling, while none of the other $CVID_i$ and $CVID_c$ patients had reported significant infections in three-month period prior to sampling.

Nine $CVID_c$ patients suffered from GI complications, four of these patients also suffered from GLILD and were included in the $CVID_{ild}$ together with one patient without GI complications. The remaining five patients were included in the $CVID_{gi}$ group. Three patients were treated with immunosuppressants at the time of sampling.

Figure 1: aCD3 stimulation induces proliferation of CD4+ and CD8+ central memory (CM) and effector memory (EM) cells. *Proliferation in CD4 CM (A), CD4 EM (B), CD8 CM (C) and CD8 EM (B) cells was compared after 4 days of culture between the negative control, aCD3 stimulation and stimulation with CD3/CD28 beads (Beads 1:5). Active proliferation was defined as cells that expressed diminished or no cell trace violet. Stimulation with aCD3 and with beads induced significant proliferation (Kruskal-Wallis, *p<0.05, #p<0.01) in all subsets and stimulation with beads induced more proliferation than stimulation with aCD3. There were no significant differences in proliferation between healthy controls (HC), $CVID_i$, $CVID_{gi}$ and $CVID_{ild}$*

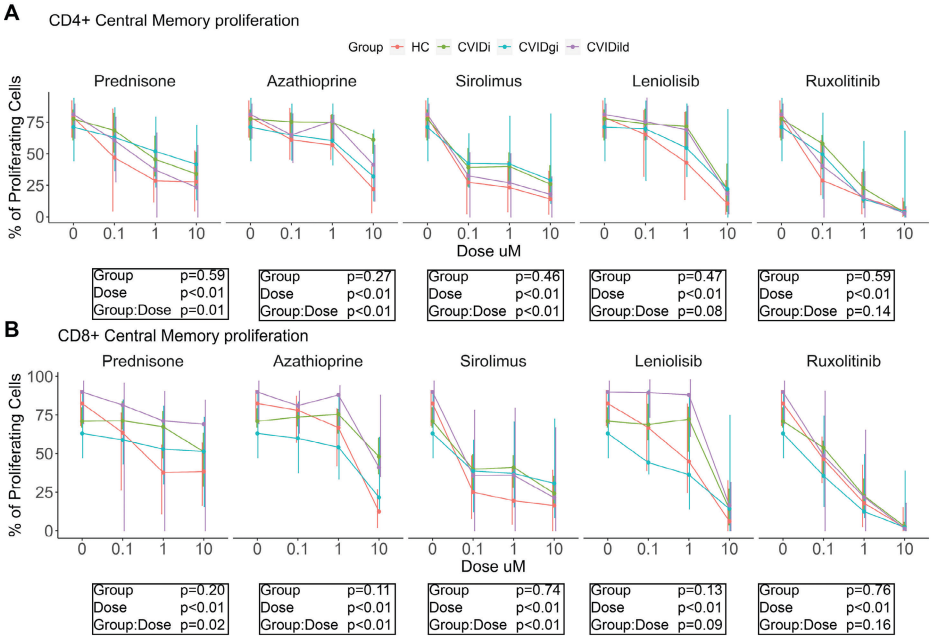


T cell proliferation

Activation with αCD3 led to proliferation of CD4+ and CD8+ CM and EM cells in all subjects (Figure 1).

We found a strong dose dependent effect for all five immunosuppressants in the CD4+ and CD8+ CM subsets in healthy controls, while the response to the five immunosuppressants varied per CVID group (Figure 2A-B). When exploring CD4+ and CD8+ EM subsets, we found similar results (Supplementary Figure 3).

Figure 2: Immunosuppressants effectively reduce proliferation in a dose dependent manner in αCD3 stimulated cells of healthy controls, but prednisone is less effective in CVID$_{gi}$ and azathioprine is less effective in CVID$_{ild}$. *Proliferation in CD4 CM (A) and CD8 CM (B) cells was compared after 4 days of culture with 0.1, 1 and 10 μM of prednisone, azathioprine, sirolimus, leniolisib and ruxolitinib and αCD3. Active proliferation was defined as cells that expressed diminished or no cell trace violet. All drugs showed a significant reduction of proliferation in a dose-dependent manner (aligned rank transformation ANOVA) and prednisone, azathioprine and sirolimus showed significant interactions between the group and dose.*

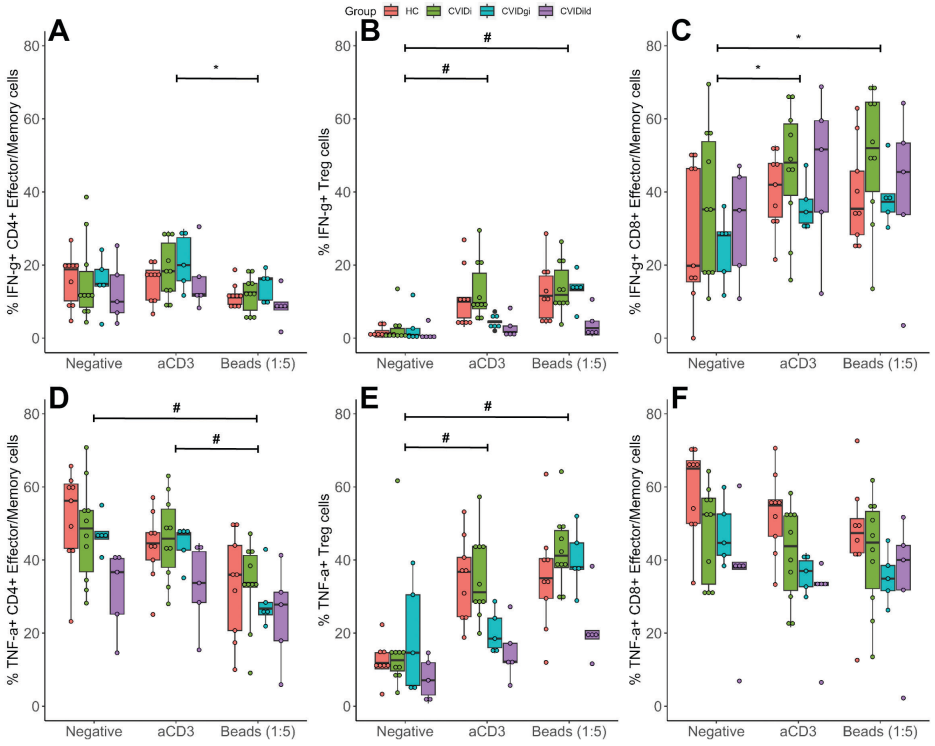


Regarding the CVID$_c$ subjects, prednisone could not effectively reduce proliferation of CD4+ CM cells in CVID$_{gi}$ patients, and CD8+ CM cells CVID$_{ild}$. Azathioprine was less effective in CVID$_{ild}$. Leniolisib reduced proliferation of CD4+ and CD8+ CM cells in all CVID patients, but only in high concentrations. Sirolimus and ruxolitinib, on the other hand, could effectively reduce proliferation of CD4+ and CD8+ CM cells at

lower concentrations, although sirolimus was potentially less effective in all CVID patients compared to healthy controls. Thus, sirolimus and ruxolitinib seemed more effective in reducing T cell proliferation for $CVID_c$.

Interestingly, $CVID_i$ patients also seemed to show a reduced response to immunosuppressants . While these patients bear no clinical signs of auto-immunity or inflammation, they showed a comparable response to immunosuppressants to $CVID_c$ patients.

Figure 3: aCD3 stimulation induces IFN-γ production in CD8 effector memory (EM) and T_{reg} cells and TNF-α production in T_{reg} cells. *IFN-γ in CD4 EM (A), T_{reg} (B) and CD8 EM (C) and TNF-α production in CD4 EM (D), T_{reg} (E) and CD8 EM (F) was compared after 2 days of culture between the negative control, aCD3 stimulation and stimulation with CD3/CD28 beads (Beads 1:5). Stimulation with aCD3 and with beads induced increased intracellular production of IFN-γ in T_{reg} and CD8 EM cells and increased production of TNF-α (Kruskal-Wallis, *p<0.05, #p<0.01). There were no significant differences in intracellular cytokine production between healthy controls (HC), $CVID_i$, $CVID_{gi}$ and $CVID_{ild}$ in the CD4 EM and CD8 EM subsets. However, T_{reg} cells of $CVID_{gi}$ and $CVID_{ild}$ produced less intracellular cytokines (pairwise-Wilcoxon, p<0.05).*



Intracellular cytokine production in CVID

Secondly, we investigated the intracellular cytokine production (Figure 3). We found that αCD3 activation induced intracellular IFN-γ production in T_{reg} and CD8 effector memory cells. Similarly, TNF-α production in T_{reg} in all groups increased. IFN-γ production in CD4 effector memory cells and TNF-α production in CD4 and CD8 effector memory cells were not affected. Patients with $CVID_{gi}$ and $CVID_{ild}$ showed less intracellular TNF-α production in CD8+ effector/memory cells in comparison to healthy controls (p=0.04). Additionally, patients with $CVID_{gi}$ and $CVID_{ild}$ showed hampered intracellular IFN-γ ($CVID_{gi}$: p=0.06, $CVID_{ild:}$ p=0.04) and TNF-α ($CVID_{gi}$: p=0.03, $CVID_{ild:}$ p=0.02) production in regulatory T cells (T_{reg}).

We found potential dose dependent repression of intracellular cytokines in T_{reg} (Figure 4), CD4 effector/memory cells (Supplementary Figure 4) and CD8 effector/memory cells (Supplementary Figure 5) . However, in the effector/memory subsets there were no clear differences in the efficacy of the immunosuppressants between the different groups and the immunosuppressants could not restore the hampered intracellular cytokine production in T_{reg} cells.

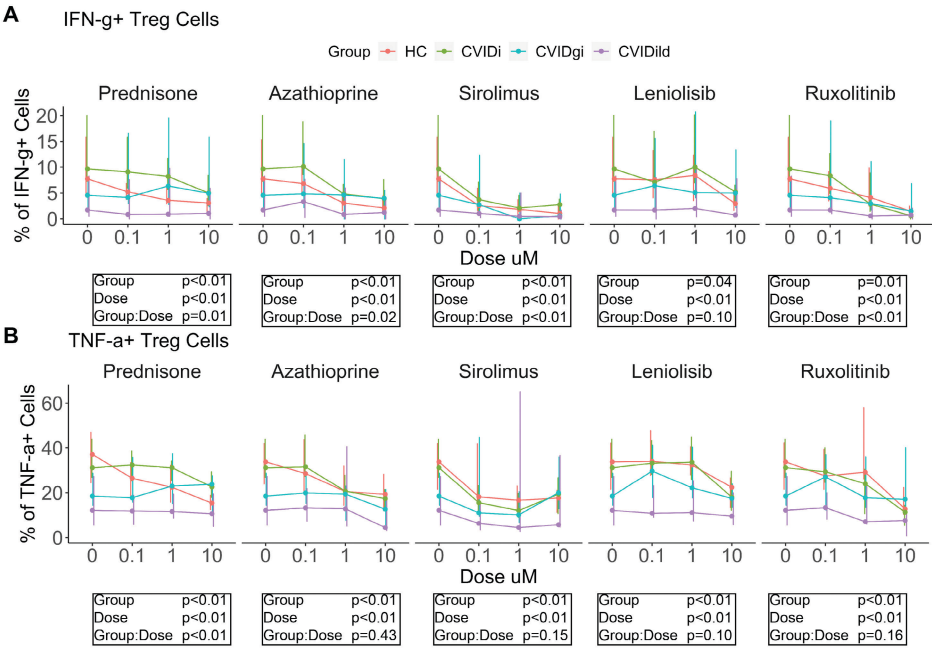
Predictive capacity of immunosuppressant efficacy

Since the proliferation assay showed more pronounced differences in comparison to cytokine production, we used these results to compare the efficacy of immunosuppressants in this assay to their clinical efficacy (Table 2).

We did not find large differences in the predictive capabilities of specific subsets. The assay predicted a correct response in 44/68 (65%) subsets when the cut-off was used and a correct response in 45/68 (66%) subsets when the Δ was used. We then investigated whether the predictive accuracy could be improved by combining subset data. We combined data of all subsets and analyzed different criteria for accuracy. The prediction was most accurate when at least 3 of the subset results matched the clinical outcome. This was the case in 13/18 (72%) cases when the cut-off was used and 11/18 (61%) cases when the Δ was used.

The efficacy of prednisone matched the clinical efficacy in 6/9 cases. In two cases a response was predicted while these patients showed no clinical signs of a complete response. Instead, a partial response was seen in only one of these patients, which led the clinician to switch to another immunosuppressive regimen. Moreover, non-response to prednisone was predicted in one patient while the patient showed clinical response.

Figure 4: Immunosuppressants reduce intracellular cytokine production in a dose dependent manner in aCD3 stimulated T_{reg} cells of healthy controls and fail to restore intracellular cytokine production in $CVID_{gi}$ and $CVID_{ild}$ T_{reg} cells. *Intracellular in IFN-γ (A) and TNF-α (B) production in T_{reg} cells was compared after 2 days of culture with 0.1, 1 and 10 μM of prednisone, azathioprine, sirolimus, leniolisib and ruxolitinib and aCD3. All drugs showed a significant reduction of intracellular cytokine production in a dose-dependent manner in healthy controls (HC) and $CVID_i$ (aligned rank transformation ANOVA), but failed to restore the hampered cytokine production in $CVID_{gi}$ and $CVID_{ild}$.*



The efficacy of azathioprine matched the clinical response in 5/6 cases. In one case non-response was predicted while the patient showed clinical response. This might be caused by the fact that this patient was treated with a combination of azathioprine and prednisone.

The efficacy of sirolimus matched the clinical response in 2/3 cases. In one case a response was predicted while the patient showed no clinical response. This was a patient that was treated with rituximab and sirolimus for GLILD and showed an initial response, but relapsed later on. The relapse could have been caused by B cell reconstitution, but this was not further investigated by the clinician.

Table 2: *Individual clinical response per immunosuppressant compared to individual response per subset in the in vitro assay.*

ID	Clinical response	CD4CM		CD4EM		CD8CM		CD8EM		Correct	
		CO: <33%	Δ: >46%	CO: <19%	Δ: >46%	CO: <37%	Δ: >45%	CO: <29%	Δ: >57%	CO	Δ
Pred:											
CVIDc1	–	–	–	–	–	–	–	–	–	4	4
CVIDc2	–	+	+	+	–	+	+	+	+	0	1
CVIDc3	–	–	–	+	+	–	–	–	–	3	3
CVIDc4	–	–	–	–	–	–	–	+	–	3	4
CVIDc6	+	–	–	–	–	–	–	+	–	1	0
CVIDc7	–	+	–	–	–	–	–	+	–	2	4
CVIDc8	–	–	–	–	–	–	–	–	–	4	4
CVIDc9	–	+	+	–	–	–	–	–	–	3	3
Aza:											
CVIDc2	–	–	–	+	–	–	–	–	–	3	4
CVIDc3	–	–	–	–	–	–	–	–	–	4	4
CVIDc4	–	–	–	–	–	–	–	–	–	4	4
CVIDc7	–	–	–	+	–	–	–	–	–	3	4
CVIDc8	+	–	–	+	+	+	–	+	+	3	2
CVIDc9	+	–	–	–	–	–	–	–	–	0	0
Sir											
CVIDc2	+	+	+	+	–	+	+	+	+	4	3
CVIDc3	–	+	+	+	+	+	+	+	+	0	0
CVIDc6	+	–	–	+	–	+	+	+	–	3	1
Correct		10	11	9	10	12	11	11	11	44	45

CM = central memory; CO = cut-off; $CVID_c$ = CVID with non-infectious complications; Δ = delta; pred = prednisone; sir = sirolimus

DISCUSSION

This study aimed to explore an *in vitro* T cell assay for prediction of individual treatment responses in $CVID_c$. We show that αCD3 induced proliferation in CD4+ and CD8+ central memory and effector memory T cells in healthy controls, $CVID_i$ and $CVID_c$. Exposure to several immunosuppressants could differentially suppress this proliferation. Prednisone was less effective at inhibiting T cell proliferation in $CVID_{gi}$ patients, whilst azathioprine was less effective at inhibiting T cell proliferation in $CVID_{ild}$. Furthermore, we show that αCD3 stimulation induces IFN-γ production

in T_{reg} and CD8+ effector/memory cells in healthy controls, $CVID_i$ and $CVID_c$ and that IFN-γ and TNF-α production is hampered in $CVID_c$ T_{reg} cells. Even though intracellular cytokine production could be inhibited with immunosuppressants, we did not find any differences in the efficacy among groups. Finally successful *in vitro* inhibition of T cell proliferation of at least 3 out of 4 T cell subsets showed to match with clinical response in 72% of cases.

Varying effectivity of prednisone in $CVID_{gi}$, as we show here in our *in vitro* assays, has previously been reported,[3] while the effectivity of sirolimus in $CVID_{gi}$ has not yet been described. Interestingly, ruxolitinib was the only drug in our assay that could effectively reduce CD4+ and CD8+ subset proliferation in $CVID_{gi}$. An upregulation of type I interferon response genes has previously been shown for $CVID_{gi}$ and the use of JAK inhibitors like ruxolitinib has been propagated.[35] This assay suggests that ruxolitinib is more efficient for suppression of T cell mediated inflammatory disease than other immunosuppressants in $CVID_{gi}$.

Varying effectivity of prednisone, has recently been described in $CVID_{ild}$.[14] Effectivity of azathioprine and sirolimus monotherapy has been incidentally reported, while effectivity of combination therapy of rituximab and azathioprine has clearly been shown *in vivo* in larger patient series.[4,16] In our assay, azathioprine monotherapy seemed less effective than prednisone and sirolimus, suggesting that it is less favorable as steroid-sparing agent. Moreover, ruxolitinib was also efficient in reducing proliferation and restoring cytokine production in $CVID_{ild}$, suggesting that ruxolitinib could be used as alternative therapy when other treatment regimens fail.

In our assay, combined T cell subset proliferation was associated with a clinical response and could therefore potentially be used as a predictor of clinical response. The relation between T cell proliferation and clinical response to immunosuppressants has been previously shown for kidney transplant recipients.[30] Here, researchers showed a correlation between the *in vitro* efficacy of immunosuppressants on T cell proliferation and graft rejection status, using activated PBMC. Additionally, the accuracy of these assays could potentially still be improved by matching the timing of clinical exposure to immunosuppressants and the timing of sampling. Ideally, these *in vitro* assays would be performed right before clinical exposure to the drug

Moreover, both assays analyzed the proliferative capacity of T cells in the PBMC fraction to predict the response of T cells in the tissue inflammatory

microenvironment. A drawback of this approach is that the phenotype of T cells from the PBMC fraction might not reflect the phenotype of T cells from the tissue inflammatory microenvironment and response prediction might therefore be inaccurate. Moreover, both assays only looked at T cells, while the immunosuppressants that were used also affect the other immune cells in the PBMC fraction. An *in vitro* assay that uses immune cells isolated from tissue biopsies might therefore more accurately model the clinical response to immunosuppressants.

Still, using the PBMC fraction has multiple advantages. First, it doesn't require an invasive procedure with a considerable risk of complication. Previous research has found an overall complication rate of 5.2% and complications occurred in 17.1% of intrathoracic biopsies.[36] This demonstrates that repetitive biopsies, especially for pulmonary diseases like GLILD, are not preferable. Second, isolating sufficient T cells from a biopsy can be challenging and requires advanced and time-consuming culturing techniques,[37] while PBMC can be obtained in greater number and are available for culture directly ex-vivo. Finally, procedures to acquire, isolate, store and culture PBMC are widely used, relatively inexpensive and scalable and other assays that use PBMC have already been clinically implemented.[38,39] This makes successful clinical implementation of an in vitro assay that uses PBMC more likely.

In this study, intracellular cytokine production was not an accurate predictor for clinical response in CVID. This could be caused by the fact that $CVID_c$ patients showed little differences in intracellular cytokine production and longer exposure to immunosuppressants might be required to normalize these minor differences.

Moreover, other limitations also need to be considered when interpreting the results of our study. First, the low sample size of our study could have introduced type 1 errors. This seems unlikely, however, since the results of our study also are similar to clinical evidence described in previous research. Second, three $CVID_c$ patients were actively treated with immunosuppressants during sampling. Despite immunosuppressant therapy, these three patients still showed individual variation on different immunosuppressants *in vitro*. However, it would be more optimal to perform this assay before immunosuppressive therapy has been started, as it makes the results of the assay easier to interpret. Third, patients with $CVID_i$ showed a similar response to immunosuppressants as $CVID_c$ patients. This could, however, be caused by the fact that broad T cell activation through αCD3 stimulation, reflects a pathophysiologic mechanism similar to $CVID_c$. Fourth, this assay included 10 µmol concentrations of immunosuppressants, which potentially do not reflect

concentrations that are achievable *in vivo*. Finally, due to the experimental nature of this assay, clear cut-offs that define success of immunosuppressive therapy have not yet been established and multiple definitions of response accuracy were tested, potentially biasing the previously reported accuracy rates.

We conclude that this *in vitro* assay, measuring proliferation of T cell subsets after exposure to immunosuppressive medication, might potentially serve as predictor of clinical response in $CVID_c$. Clinical application of this assay might be feasible, although future research that identifies clear cut off values for the read out validates the use of this assay prospectively are still required.

Acknowledgements
We would like to thank the patients for their participation in this study. We would also like to thank the Louise Vehmeijer foundations, the Dirkzwager Assink Fund and de Merel Foundation for their financial support.

Disclosures
Our funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The investigators involved in this manuscript have no potentially conflicting financial interests to disclose.

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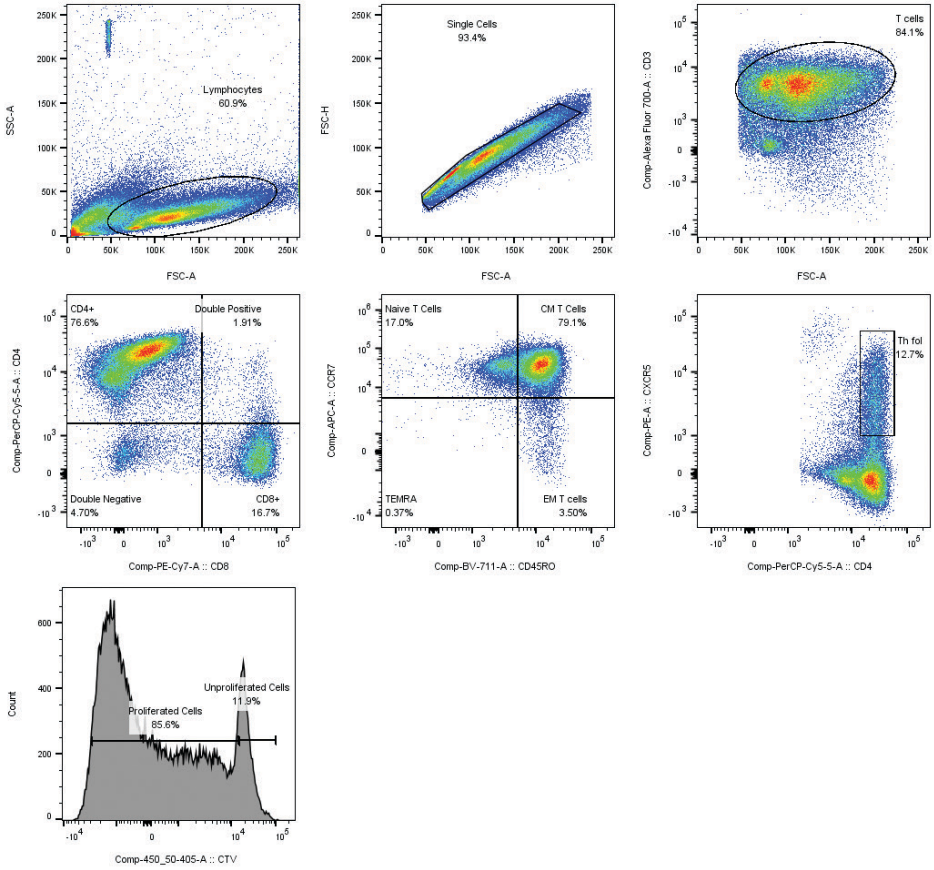
SUPPLEMENTARY TABLES

Supplementary Table 1: *Antibodies used for FACS staining*

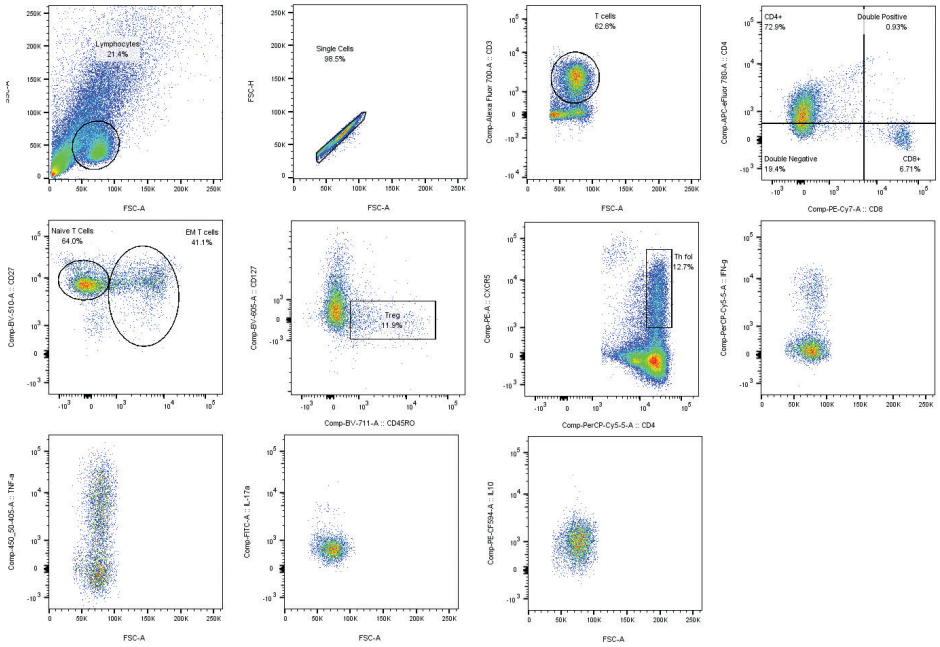
Panel	Intracellular/ Surface	Target Name	Fluorchrome	Clone	Supplier	Cat #
Cytokine Production	Surface	CD3	AF700	UCHT1	Biolegend	300424
	Surface	CD4	APC-eFluor780	RPA-T4	Antibody Chain	47-0049-42
	Surface	CD27	BV510	L128	BD	563092
	Surface	CD127	BV605	A019D5	Antibody Chain	RT2356670
	Surface	CD45RO	BV711	UCHL1	Biolegend	304236
	Surface	CD8	PE-Cy7	SK1	BD	335822
	Intracellular	IL-17(a)	FITC	eBio64DEC17	eBioscience	11-7179-42
	Intracellular	IFN-g	PerCP-CY5.5	4S.B3	Biolegend	502526
	Intracellular	FoxP3	APC	PCH101	Thermo Fisher Scientific	11560617
	Intracellular	TNF-a	BV421	MAb11	BD	566275
	Surface	CXCR5	PE	J252D4	Biolegend	356904
	Intracellular	IL-10	PE-CF594	JES3-19F1	BD	562400
Proliferation	Surface	CD28	FITC	CD28.2	eBioscience	11-0289-42
	Surface	CD4	PerCP-CY5.5	SK3	BD	566923
	Surface	CCR7	APC	G043H7	Biolegend	353214
	Surface	CD3	AF700	UCHT1	Biolegend	300424
	Surface	CD27	BV510	L128	BD	563092
	Surface	CD45RO	BV711	UCHL1	Biolegend	304236
	Surface	CXCR5	PE	J252D4	Biolegend	356904
	Surface	CD8	PE-Cy7	SK1	BD	335822

SUPPLEMENTARY FIGURES

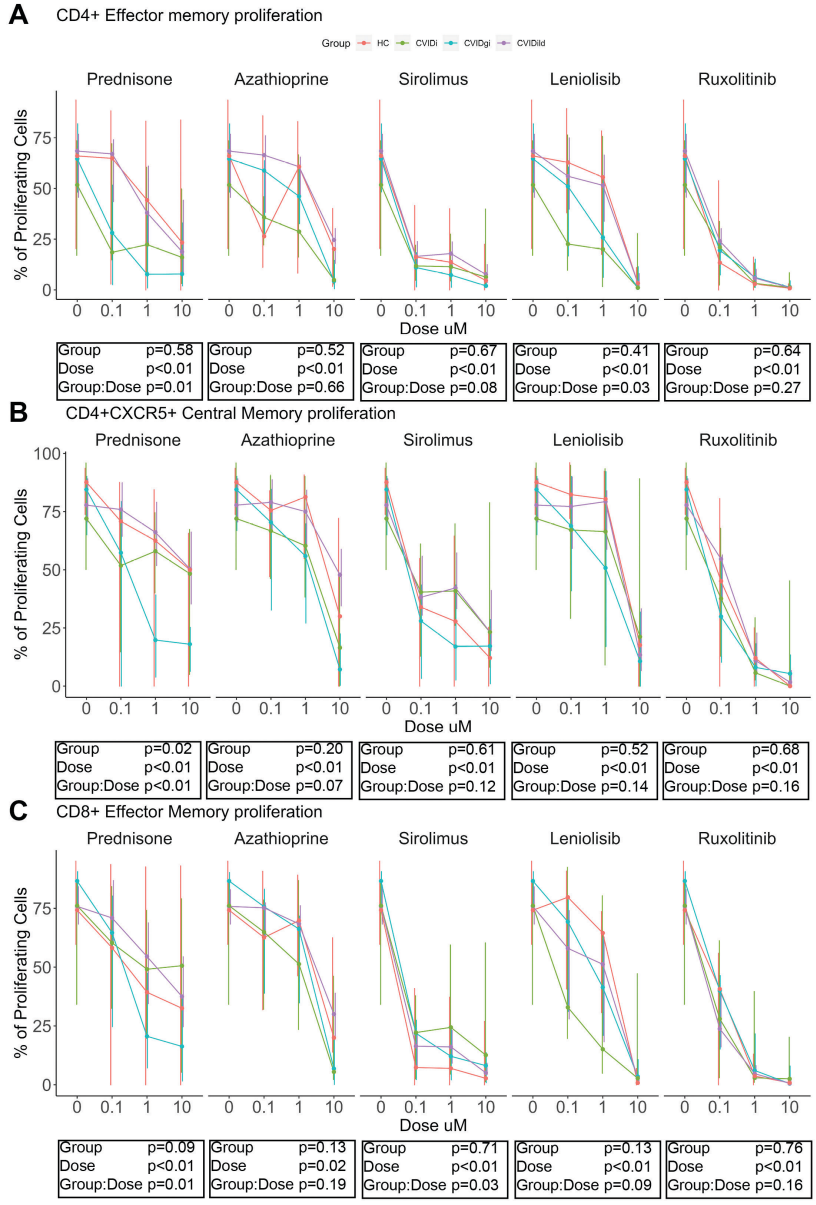
Supplementary Figure 1: *Gating strategy for the proliferation panel*



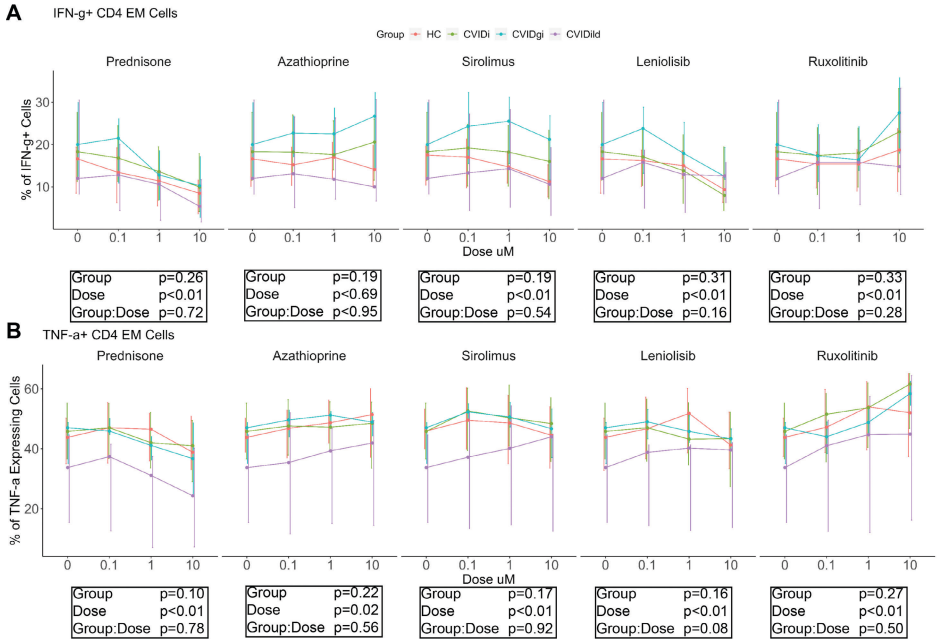
Supplementary Figure 2: *Gating strategy for the intracellular cytokine production panel*



Supplementary Figure 3: Immunosuppressants effectively reduce proliferation in a dose dependent manner in αCD3 stimulated cells. of healthy controls. *Proliferation in CD4 EM (A), CD4 CXCR5+ CM (B) and CD8 EM (C) cells was compared after 4 days of culture with 0.1, 1 and 10 μM of prednisone, azathioprine, sirolimus, leniolisib and ruxolitinib and αCD3. Active proliferation was defined as cells that expressed diminished or no cell trace violet. All drugs showed a significant reduction of proliferation in a dose-dependent manner (aligned rank transformation ANOVA) and prednisone and sirolimus showed significant interactions between the group and dose. Thus, prednisone and sirolimus seemed less effective in CVID$_{ild}$.*

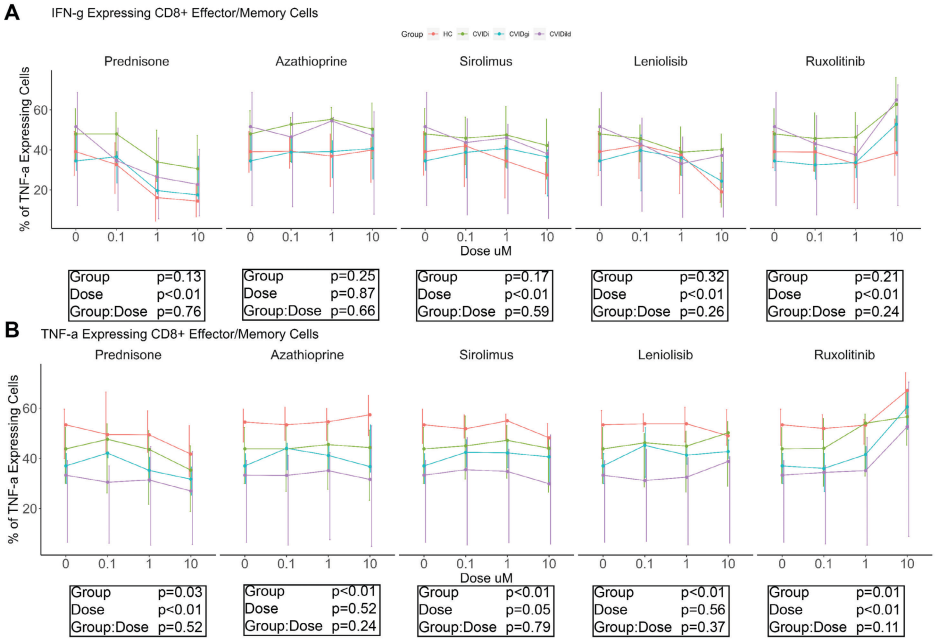


Supplementary Figure 4: Immunosuppressants reduce intracellular IFN-γ and induce intracellular TNF-α production in a dose dependent manner in αCD3 stimulated in CD4 effector/memory cells
Intracellular in IFN-γ (A) and TNF-α (B) production in CD4+ effector memory (EM) cells was compared after 2 days of culture with 0.1, 1 and 10 μM of prednisone, azathioprine, sirolimus, leniolisib and ruxolitinib and αCD3. Azathioprine and ruxolitinib did not reduce intracellular IFN-γ production in a dose-dependent manner (aligned rank transformation ANOVA).



Supplementary Figure 5: Immunosuppressants reduce intracellular IFN-γ and induce intracellular TNF-α production in a dose dependent manner in αCD3 stimulated in CD8 effector/memory cells

Intracellular in IFN-γ (A) and TNF-α (B) production in CD8+ effector memory (EM) cells was compared after 2 days of culture with 0.1, 1 and 10 μM of prednisone, azathioprine, sirolimus, leniolisib and ruxolitinib and αCD3. Azathioprine and ruxolitinib did not reduce intracellular IFN-γ production in a dose-dependent manner, while azathioprine and leniolisib did not induce intracellular TNF-α production in a dose-dependent manner (aligned rank transformation ANOVA).

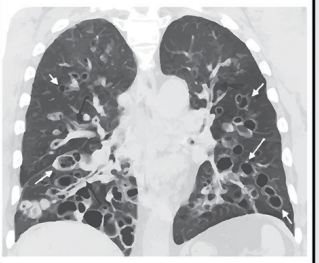


PART II:

Management of PAD

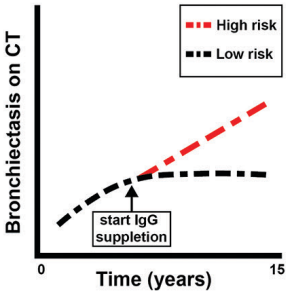
Patients with primary antibody deficiency were retrospectively included:

- XLA (n = 11)
- CVID (n = 54)
- IgSD/SPAD (n = 18)
- unPAD (n = 8)



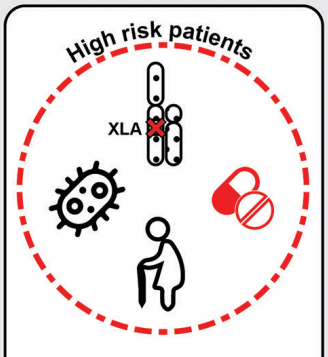
Patients had been screened regularly for pulmonary disease with low-dose CT.

Pulmonary disease was quantified using the Brody II score.



Bronchiectasis stabilized after start of IgG suppletion in low-risk patients.

However, in high-risk patients bronchiectasis still progressed.



High risk patients were identified by:

- BTK deficiency
- Prophylactic antibiotics
- Older age
- Frequent infections

CHAPTER 6

Pulmonary screening frequencies can potentially be reduced in low-risk primary antibody deficiency.

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ABSTRACT

Background: Patients with primary antibody deficiency (PAD) frequently suffer from pulmonary complications, associated with severe morbidity and mortality. Hence, regular pulmonary screening by computed tomography (CT) scanning is advised. However, predictive risk factors for pulmonary morbidity are lacking.

Objective: To identify PAD patients at risk for pulmonary complications necessitating regular CT screening.

Methods: A retrospective longitudinal cohort study of PAD patients (median follow-up 7.4 (2.3 – 14.8) years). CTs were measured using the modified Brody-II scoring system. Predefined potential risk factors were collected.

Results: The following independent risk factors for progression of airway disease (AD) were identified: 1) diagnosis of X-linked agammaglobulinemia (XLA), 2) recurrent airway infections (2.5/year), 3) presence of AD at baseline. Signs of AD progression were detected in 5/11 XLA patients and in 17/80 of the other PAD patients. Of the patients who progressed, 17/22, had preexistent AD scores ≥7. Increased AD scores were related to poorer FEV1 values and chronic cough. CVID and increased CD4 effector/memory cells were risk factors for an interstitial lung disease (ILD) score ≥13. ILD≥13 occurred in 12/80 patients. Signs of ILD progression were detected in 8/80 patients and 4/8 patients showing progression had preexistent ILD scores ≥13.

Conclusion: We identified risk factors that distinguished PAD patients at risk for AD and ILD presence and progression which could guide future screening frequency, however independent and preferably prospective validation is needed.

HIGHLIGHT BOX

1. What is already known about this topic?

PAD patients, in general, frequently suffer from Airway Disease and Interstitial Lung Disease, associated with severe morbidity and mortality. However, predictive factors to identify the individual patients at risk for these pulmonary complications are lacking.

2. What does this article add to our knowledge?

This study identifies risk factors that can distinguish specific PAD patients at risk for Airway Disease and Interstitial Lung Disease presence and progression.

3. How does this study impact current management guidelines

Current study provides new advice regarding pulmonary screening frequencies in patients with primary antibody deficiency, specified by risk for pulmonary complications.

Key Words: Primary Antibody Deficiency; CVID; Airway Disease; GLILD; Brody II Score; Risk Factor

ABREVIATIONS

AD = airway disease
CT = computerized tomography
CVID = common variable immunodeficiency
FEV1 = forced expiratory volume in 1 second
GLILD = granulomatous interstitial lung disease
IgSD = Immunoglobulin G subclass deficiency
ILD = interstitial lung disease
PAD = primary antibody deficiency
SPAD = specific polysaccharide antibody deficiency
unPAD = unclassifiable primary antibody deficiency
XLA = x-linked agammaglobulinemia

INTRODUCTION

Primary antibody deficiency (PAD) is the most common group of primary immunodeficiencies and can be categorized into immunoglobulin subclass deficiency (IgSD), specific polysaccharide antibody deficiency (SPAD), common variable immunodeficiency (CVID), congenital agammaglobulinemia (such as X-linked agammaglobulinemia) and unclassifiable PAD (unPAD).[1–3] PAD is frequently complicated by pulmonary disease, which can be categorized into airway disease (AD) and granulomatous lymphocytic interstitial lung disease (GLILD) and which may cause high morbidity and mortality.[4]

AD is caused by recurrent lower respiratory tract infections and the subsequent structural damage, and is characterized by bronchial wall thickening, bronchiectasis and signs of mucus plugging on computed tomography (CT).[5–9] Clinical manifestations of AD are chronic (productive) cough with recurrent respiratory exacerbations and dyspnea by exertion, but early disease may go unnoticed.[9,10] Bronchiectasis represents the most severe manifestation in the spectrum of AD.[11]

Bronchiectasis is diagnosed in 34% of CVID patients and is associated with chronic sinusitis, pneumonia and decreased pulmonary function.[6,12–14] Thus, the presence of AD and specifically of bronchiectasis can lead to a downward spiral where patients increasingly suffer from pulmonary infections, leading to accumulation of lung tissue damage and chronic local inflammation. In turn, this results in more hospitalizations, decreased quality of life,end-stage pulmonary failure and eventually may lead to early death.[4,6,9,13–16]

GLILD is caused by immune dysregulation and can be characterized by nodules, ground-glass opacities and reticulation on CT.[6,7,9,16] The clinical manifestations of GLILD are chronic (dry) cough and exertional dyspnea. GLILD is diagnosed in 10% - 20% of CVID patients and AD and GLILD often co-occur.[17–19] GLILD has been associated with a ≥50% reduction of life expectancy due to evolvement to end-stage pulmonary failure and can be part of a systemic immune dysregulation disorder that may include, systemic granulomatous disease, splenomegaly, diffuse lymphoproliferation and auto-immune cytopenias.[17,19–22]

Earlier research showed that bronchiectasis and AD may be caused by the cumulative effect of recurrent pulmonary infections.[4,7,9,12,23] However, bronchiectasis

was also found in CVID patients who did not experience lower respiratory tract infections.[7] Furthermore, the severity of PAD, expressed as poorer B cell functionality, was associated with bronchiectasis and more respiratory complications. [13,14,23–26] One study found that AD progression was observed in patients with IgG trough levels <10 g/L during immunoglobulin replacement therapy (IRT).[27] Moreover, bronchiectasis may also be the result of recurrent micro aspiration caused by chronic recurrent sinusitis. [28]

Risk factors for GLILD have been studied less, but GLILD has been associated with autoimmune cytopenia and splenomegaly, lower IgG and IgA levels at diagnosis, reduced class switched memory B cells and increased $CD21^{low}$ B cells and reduced pulmonary function in CVID patients.[7,19,25,29]

Despite these previous efforts, it remains unclear if one single risk factor is responsible for AD progression or if the causes are multifactorial. Moreover, earlier studies mostly investigated risk factors associated with the presence of bronchiectasis without taking the severity into account, which might bias risk factor prediction outcomes. Additionally, it is unclear if risk factors for AD progression are similar for all PAD subtypes, since associations between bronchiectasis and PAD classification have been shown. [12,14,23] Finally, publications about risk factors for ILD development have been scarce, with small sample size and relatively short follow-up times. In this study, we analyze CT scans of PAD patients during a follow up time of 7.4 years (2.3-14.8 years) and aim to identify which factors can adequately identify PAD patients at risk for AD and GLILD progression across the different types of PAD.

METHODS

Study population

PAD patients in care at the University Medical Center Utrecht between 2008 and 2021 were screened for pulmonary disease at regular intervals using chest CT.

Study design

We conducted a non-interventional, single center retrospective cohort study. Retrospective documentation started at the first CT screening event. Secondary clinical and lab parameters recorded 12 months prior or after CT screening date were retrospectively collected from the patients' records.

Eligibility

Patients were included if they met the following criteria:

- A diagnosis of XLA, CVID, IgSD and/or SPAD according to the European Society for immune deficiencies criteria or an unclassifiable hypogammaglobulinemia (unPAD).
- Active immunoglobulin replacement therapy.
- Availability of at least two CT scans with a minimum interval of two years.
- No active treatment for GLILD during the study

All CT scans had been performed routinely as part of the standard care screening protocol in our hospital or because of a clinical indication. Most participants in this study already participated in a broad observational review board–approved study (National PID study, METC: NL40331.078) for which they had provided written consent. The remaining participants provided their consent for use of medical data and CT images.

CT screening and scoring

We used a previously described routine protocol for CT. Scans were acquired during inspiration and expiration. All scans were volumetric and reconstructed with thin slices. Dose was maintained as low as possible by adapting kilovoltage and milliamperes to patient size. The presence of structural pulmonary disease was scored by an independent observer according to the modified Brody-II method, used in previous studies.[7,27] Signs of AD were scored by assessing the presence of bronchiectasis, airway wall thickening, mucus plugging, tree-in-bud and air-trapping. Signs of ILD were scored by assessing the presence of opacities, ground glass, septal thickening and lung nodules. Sum scores for AD and ILD were calculated and used as primary parameter. In this study we defined, an AD score ≥7 and an arbitrary AD score increase of >0.5/year as clinically significant.[7]

Secondary parameters

Secondary parameters consisted of clinical, laboratory and pulmonary parameters recorded during regular out-patient visits and are listed in Supplementary Table 1. Data recorded 12 months prior to and up to 12 months after CT screening dates were used. Time until PAD diagnosis was defined as the time (years) between year of onset of disease-related symptoms and year of PAD diagnosis. Cough and progressive dyspnea on exertion were defined as clinical symptoms of pulmonary disease. Cough that lasted longer than 8 weeks was defined as chronic cough. Continuous variables were included if they were collected at least once during follow-up.

Statistical analysis

Continuous, non-normally distributed variables were analyzed using Kruskal-Wallis test or Mann-Whitney U test as appropriate. Chi-squared tests and Fisher's exact tests were performed for categorical variables as appropriate. Time-dependent data were analyzed with generalized linear models, when assumptions of linearity and distribution were met. First, medians of continuous variables were calculated and risk factors were analyzed using the previously described univariable methods. Variables with a $p<0.2$ were selected for multivariable risk factor analysis. Multiple imputation was applied to account for missing data when <40% of the original data was missing. Variables were excluded when >40% of the original data was missing. Since outcome variables were binominal in nature and the dataset contained both continuous and categorical predictors, multivariable logistic regression was used to analyze the remaining risk factors. Significance was reached when $p<0.05$. Cut-offs were calculated, requiring in minimum sensitivity of 80% and sensitivity and specificity of combined risk factors were calculated. Moreover, a cut-off for AD scores was calculated that could predict the presence of chronic cough using a minimum sensitivity of 80% as prerequisite. R Studio version 4.2.1 was used for data analysis.

RESULTS

Study Population

We included 91 patients (11 XLA, 54 CVID, 18 IgSD/SPAD and 8 unPAD patients) with a median follow-up of 7.4 years (2.3 – 14.4 years). At inclusion, the median age was 26.5 years (7 – 71 years) 32 patients were children. At inclusion, the median duration of PAD related symptoms was 13 years (0 – 47 years) and the median diagnostic delay was 5.5 years (0 – 39 years). XLA patients tended towards longer duration of PAD related symptoms and a shorter diagnostic delay (Table 1).

Clinical, pulmonary and laboratory parameters

Clinical, laboratory and pulmonary parameters are shown in Table 1. We found that immunoglobulin replacement therapy (IRT) dose was higher in unPAD patients than in CVID and XLA patients. We found no difference in the proportion of patients that smoked, had asthma or chronic obstructive pulmonary disease, nor in the frequency of pulmonary symptoms between the different PAD groups. We found significantly more non-infectious complications in CVID patients, specifically more GLILD, which was not reported in the other PAD groups. Despite this difference we found no difference in immunosuppressant treatment between the different groups. We could not compare baseline immune subsets between the different PAD groups because of missing data.

Table 1: XLA patients had a different distribution of potential identifying factors for increased AD scores and AD progression. *We found that male sex and underlying genetic variants were more frequent in XLA. Moreover, there was less diagnostic delay and there were less non-infectious complications among XLA patients. Furthermore, immunoglobulin levels were lower in CVID patients, than in IgSD/ SPAD patients and unPAD patients, whilst IRT dosing was higher in unPAD patients, than in CVID and XLA patients.*

	XLA	CVID	IgSD/ SPAD	unPAD
n	11	54	18	8
General parameters				
Age in years (IQR)	24.5 (16.3)	31.5 (24.8)	20 (20)	13 (28.8)
% Males (N)	100 (11)	50 (27)	50 (9)	50 (4)
% Genetic variant (N)	100 (11)	38 (8)	50 (3)	17 (1)
Years of PAD related symptoms (IQR)	23.5 (14)	11 (9)	14.5 (12)	10.5 (9)
Diagnostic delay of PAD (IQR)	2 (3.5)	5 (7)	8.5 (9.3)	9 (9)
Years of follow-up (IQR)	5.5 (2.2)	8.3 (3.6)	6.3 (4.8)	6.1 (3.9)
Average number of CTs performed during follow-up	3.1	2.6	3	2.7
Pulmonary status				
% Current smoker (N)	0 (0)	23 (10)	24 (4)	25 (2)
% Asthma (N)	0 (0)	15 (8)	11 (2)	13 (1)
% COPD (N)	0 (0)	2 (1)	0 (0)	25 (2)
% Incidental cough (N)	27 (3)	28 (15)	28 (5)	25 (2)
% Chronic cough (N)	36 (4)	31 (17)	17 (3)	38 (3)
% Dyspnea (N)	0 (0)	13 (7)	6 (1)	13 (1)
Infectious Complications				
Patient reported use of antibiotic courses per year (IQR)	0.97 (0.9)	0.7 (0.7)	0.92 (0.7)	1.64 (1.7)
% Prophylactic antibiotics (N)	45 (5)	46 (25)	33 (6)	75 (6)
Prophylactic antibiotics (months/year, IQR)	3.8 (3)	5 (4)	8.5 (2)	8.2 (5)
Infections per year (IQR)	0.95 (0.8)	0.68 (0.7)	0.92 (0.7)	1.21 (1.4)
IRT dose (g/kg/week, IQR)	0.11 (0.03)	0.12 (0.04)	0.14 (0.06)	0.17 (0.08)
Non-infectious complications				
% Non-infectious complications (N)	9 (1)	33 (18)	6 (1)	13 (1)
% GLILD (N)	0 (0)	24 (13)	0 (0)	0 (0)
% Auto-immune cytopenias (N)	0 (0)	13 (7)	0 (0)	0 (0)
% Other auto-immune disease (N)	0 (0)	19 (10)	6 (1)	13 (1)
% Enteropathy/IBD (N)	9 (1)	19 (10)	0 (0)	0 (0)
% Lymphoproliferation (N)	0 (0)	17 (9)	0 (0)	0 (0)
% Malignancies (N)	0 (0)	6 (3)	0 (0)	0 (0)
% Treated with immunosuppressants (N)	27 (3)	31 (17)	0 (0)	25 (2)
Length of immunosuppressive therapy (months/year, IQR)	2.5 (5)	2.9 (7)	0 (0)	11.8 (2)

Table 1: Continued from previous page.

	XLA	CVID	IgSD/ SPAD	unPAD
n	11	54	18	8
Laboratory parameters at baseline				
IgG through levels (g/L) (IQR)	9.4 (3.4)	8.2 (3.3)	10.1 (4.0)	9.1 (4.5)
IgA (g/L) (IQR)	0.3 (0)	0.3 (0.4)	1.2 (0.7)	0.6 (0.4)
IgM (g/L) (IQR)	0.2 (0)	0.3 (0.6)	0.9 (0.4)	0.5 (0.6)
CD3+ cells (10^9/L) (IQR)	1.4 (0)	1.3 (1.1)	1.3 (0.4)	1.5 (0)
CD3+CD4+ cells (10^9/L) (IQR)	0.8 (0)	0.7 (0.6)	0.7 (0.3)	0.9 (0)
CD3+CD8+ cells (10^9/L) (IQR)	0.6 (0)	0.6 (0.4)	0.5 (0.3)	0.5 (0)
CD19+ cells (10^9/L) (IQR)	0 (0)	0.2 (0.3)	0.3 (0.1)	0.3 (0)
CT scores at baseline				
AD score (IQR)	7 (11.5)	7 (10.1)	2 (7)	3 (6)
ILD score (IQR)	6 (4.5)	4 (7)	2 (4)	2 (7)

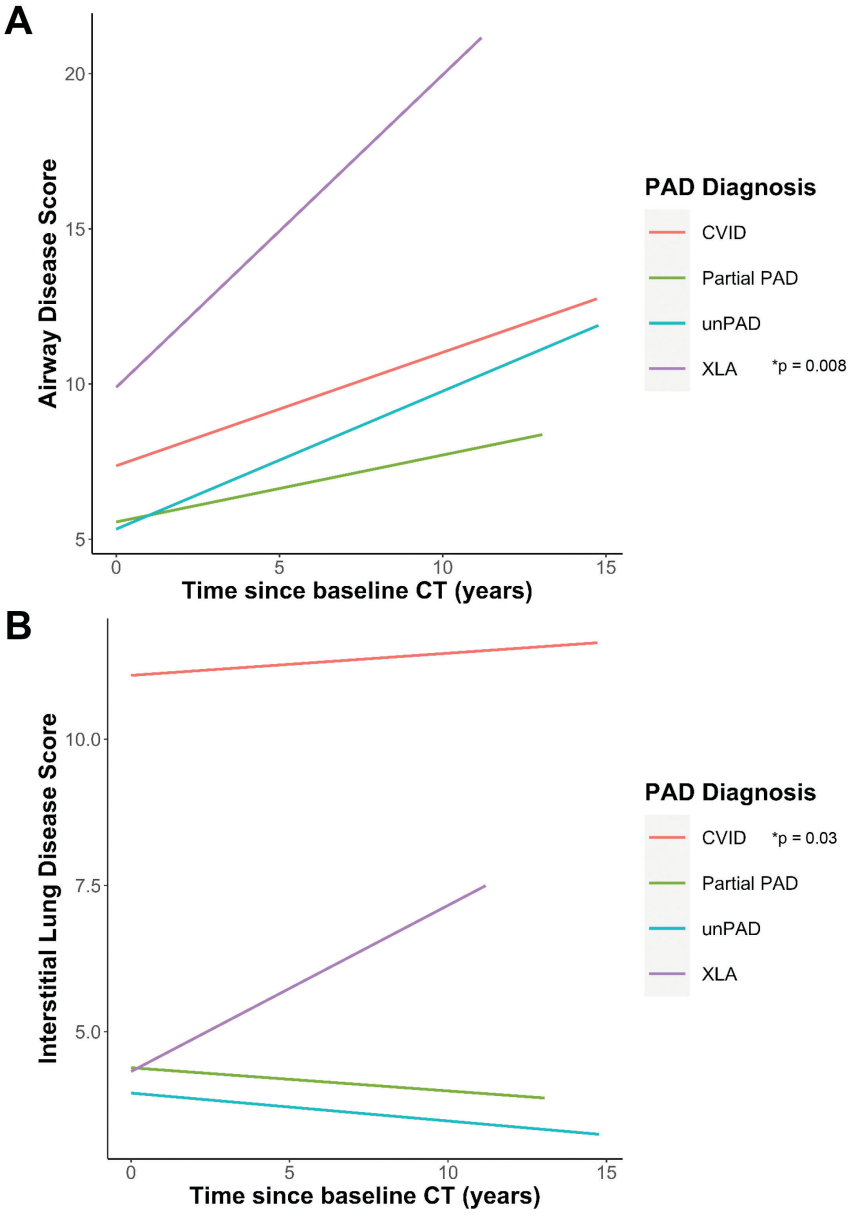
AD = airway disease, CVID = common variable immunodeficiency, FEV1 = forced expiratory volume in 1 minute, IgSD = immunoglobulin subclass deficiency, ILD = interstitial lung disease, IRT = immunoglobulin replacement therapy, PAD = primary antibody deficiency, SPAD = specific polysaccharide antibody deficiency, unPAD = unclassified primary antibody deficiency, XLA = x-linked agammaglobulinemia.

CT parameters

We compared AD and ILD progression between the different groups over time and found that AD scores were significantly higher in XLA patients (5.08 [95% CI: 1.07 – 9.12], Figure 1). Additionally, ILD scores were significantly higher in CVID patients (5.92 [95% CI: 0.62 – 11.22], Figure 1).

Our previous findings suggest that potential risk factors for AD might be different in XLA and we therefore studied the XLA patients as a separate subgroup. Finally, no signs of GLILD were detected on CT scans among XLA patients. Therefore, we did not perform a subgroup analysis for ILD risk factors in XLA.

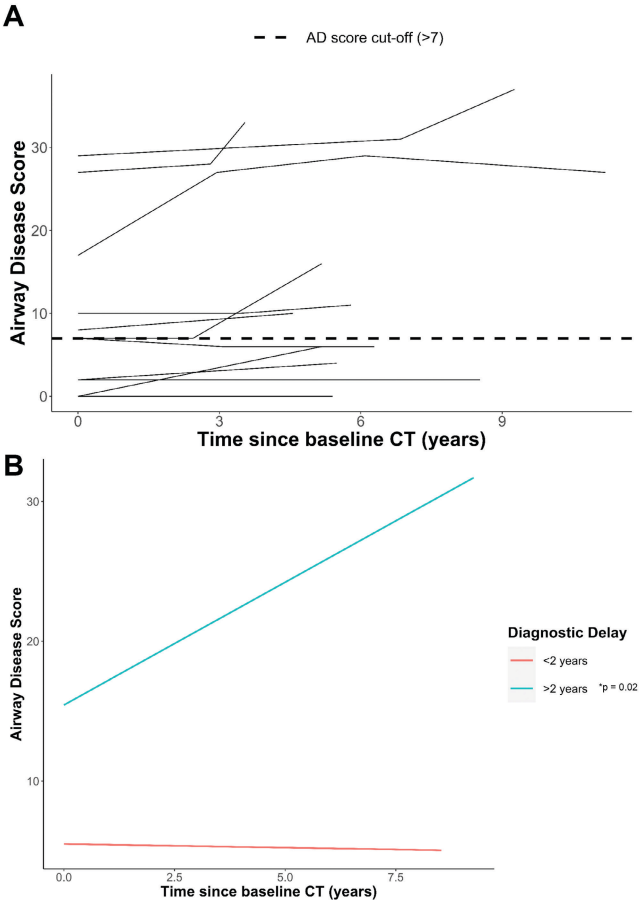
Figure 1: Airway disease (AD) scores were increased in XLA patients and interstitial lung disease scores were increased in CVID patient compared to other PAD patients. *(A) AD scores increased significantly over time on follow-up CT scans(p=0.005) and were higher in XLA patient. (B) ILD scores did not increase significantly over time, but were significantly higher in CVID patients.*



Airway disease in XLA

AD scores were stable during follow-up in more than half of the XLA patients (Figure 2), we thus used median AD scores to investigate identifying factors for increased AD scores (AD-score of ≥7). Increased AD scores were present in 6/11 XLA patients for which no identifying factors were found in univariate analysis. AD progression occurred in 5/11 XLA patients, which tended to be more frequent than in the other PAD patients (p=0.08). Diagnostic delay was a univariate predictor for AD progression in XLA patients (Figure 2). A diagnostic delay of >2 years could identify patients that showed AD progression later on with 80% sensitivity and 100% specificity, and resulted in higher AD scores (9.94 points [95% CI: 0.1 – 18.78]).

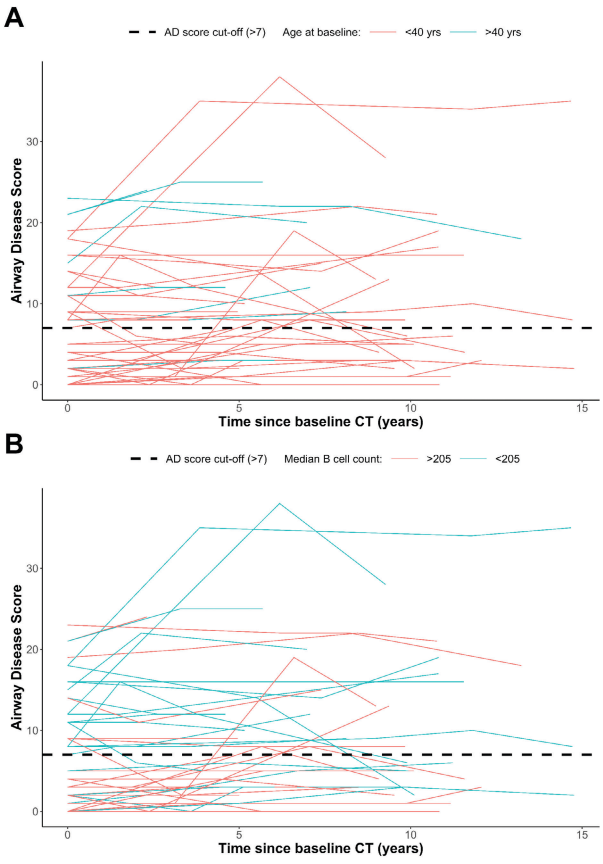
Figure 2: Diagnostic delay of the XLA diagnosis (>2 years) was a risk factor for airway disease (AD) progression in XLA patients. *A) A spaghetti plot of AD scores in XLA patients showed that 5/11 patients progressed. Patients that progressed had longer diagnostic delay. (B) Patients with a diagnostic delay >2 years had higher AD scores and potentially progressed faster (p=0.1) than the remaining XLA patients.*



Airway disease in CVID, unPAD and SPAD/IgSD

Increased AD scores were detected in 26 CVID patients, 5 IgSD/SPAD patients and 3 unPAD patients during follow-up. We found that higher age at inclusion, lower median B cell count and the presence of non-infectious complications in general, and specifically GLILD, were all significant univariate predictors. Moreover, lower median CD4 count, higher median % of CD8 effector/memory cells, lower median % of switched memory B cells, lower median IgM levels and longer diagnostic delay, were all potential univariate predictors for increased AD scores. After multivariable analysis (Electronic Supplementary Table 2), age at baseline and median B cell count were identifying factors for increased AD scores (Figure 3). Cut-offs were calculated and we found that age ≥40 years at inclusion or median B cell counts ≤205 could predict AD scores ≥7 with 79.4% sensitivity and 76.1% specificity.

Figure 3: Baseline age>40 and average B cell count <205 were independent risk factors for increased AD scores. *Spaghetti plots of AD scores in the remaining PAD patients were generated. A) Patients >40 years and B) patients with median B cell counts <205 showed AD scores ≥7 more frequently.*



In previous publications, radiographically relevant AD has been reported as AD scores ≥7, but its clinical relevance is uncertain.[7]Chronic cough is an important symptom of airway disease and was reported by 23 patients during follow-up. Patients with a chronic cough had significantly higher AD scores (12.7 vs 2.7, p<0.001) and median AD scores ≥7 during follow-up could identify patients with chronic cough with 78.3% sensitivity and 68.4% specificity. Moreover, pulmonary function tests were performed in 48/80 patients. In these patients, worse forced expiratory volume in one second (FEV1, % of predicted) correlated with higher AD scores (r=-0.32, p=0.007). Combined, these results indicate that AD scores ≥7 are clinically relevant.

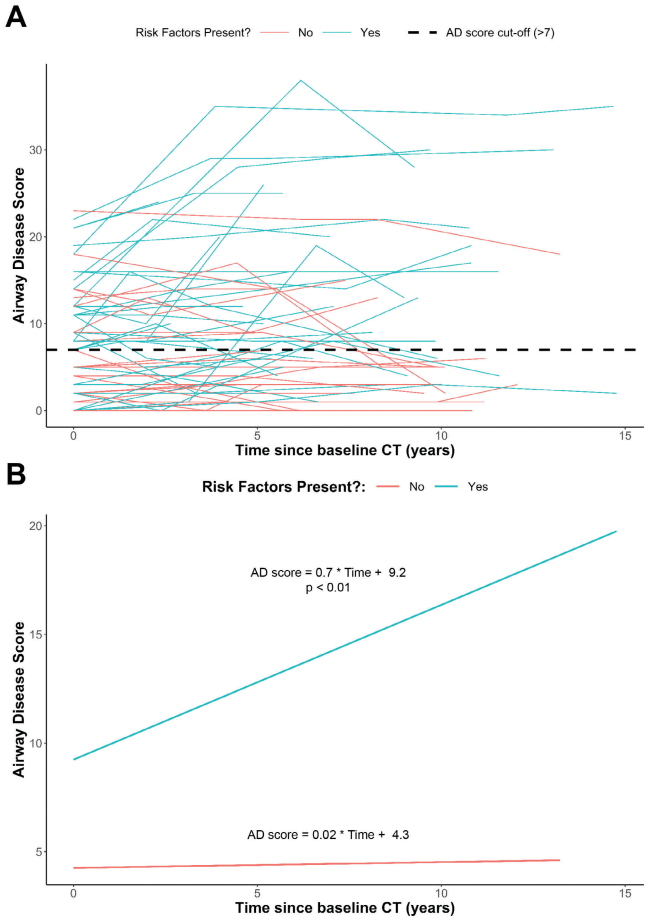
Airway disease progression in CVID, unPAD and SPAD/IgSD

AD progression was detected in only 11 of 54 CVID patients, 4 of 18 IgSD/SPAD patients and 2 of 8 unPAD patients. A higher age at inclusion, an AD score of ≥7 at baseline, more infections per year, more courses of antibiotics per year, longer prophylactic antibiotic use and the presence of non-infectious complications (specifically GLILD) were all significant univariate identifying factors for AD progression. Moreover, we found that a longer time since PAD manifestations commenced, smoking, lower CD8 counts, higher % of CD8 effector/memory cells, and higher median IRT dose were potential univariate identifying factors for AD progression. After multivariable analysis (Electronic Supplementary Table 3), the mean number of infections per year and an AD score ≥7 at baseline were predictive factors for AD progression. The total duration of prophylactic antibiotics was a potential identifying factor (Figure 3). We calculated cut-off values to identify patients with AD progression for the mean number of infections per year (2.5/year). The duration of prophylactic antibiotic therapy did not improve sensitivity or specificity. Presence of one or more identifying factors resulted in higher AD scores (4.98 points [95% CI: 2.12 – 7.8]) and faster progression (0.69 points/year [95% CI: 0.18 – 1.2], Figure 4). Combined, these risk factors could predict AD progression with 100% sensitivity and 67.4% specificity.

Risk factors for GLILD and GLILD progression

In previous publications, radiographically relevant ILD has been reported as an ILD score ≥5; however, it is not known if this is specific for GLILD.[7] The ILD score, used in this study, assessed CT related changes that can also be signs of other causes of ILD, like smoking and aging. We therefore analyzed the CT scans of PAD patients (30 scans in 13 patients) with signs of GLILD according to an independent radiologist and who had not received prior GLILD treatment. We compared these to the CT scans of PAD patients (201 scans in 67 patients) with no signs of GLILD.

Figure 4: Infections >2.5/year and AD scores ≥7 at baseline were identifying factors for patients with AD progression. *A) Individual patients with ≥2.5 infections/year and/or AD scores ≥7 at baseline (blue) were at risk for progression of AD scores. B) Patients with ≥2.5 infections/year and/or AD scores ≥7 at baseline (blue progressed while patients without risk factors did not progress.*



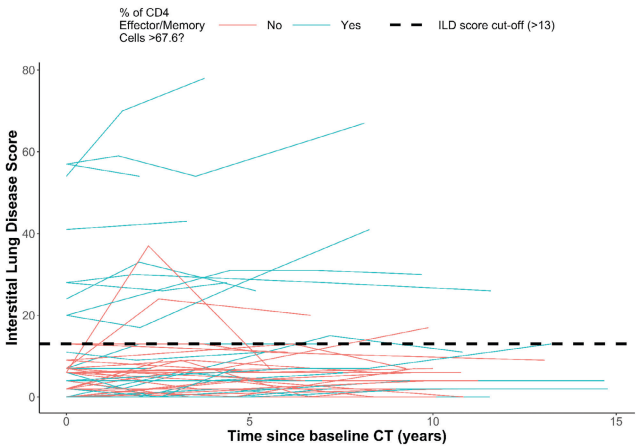
Since the ILD scores were stable during follow-up for most patients, we used the median ILD scores to analyze risk factors for relevant ILD. A median ILD score ≥5 identified CT scans that had GLILD-related abnormalities with 100% sensitivity and 68.2% specificity, while a median ILD score ≥13 identified CT scans that had GLILD-related abnormalities with 79% sensitivity and 89% specificity. Among patients diagnosed with GLILD, ILD scores ≥13 occurred on at least one CT scan in 11/13 patients.

Next, we investigated potential identifying factors for an ILD score ≥13. ILD scores ≥13 occurred in 11 CVID patients and 1 IgSD/SPAD patient. We found that AD scores ≥7 at

inclusion, higher % of CD4 effector/memory cells, lower % of switched memory B cells, higher IRT dose and the presence of non-infectious complications were all significant univariate risk factors for a median ILD score ≥13. Moreover, higher age at inclusion, smoking status, PAD diagnosis, higher % of CD8 effector/memory cells, lower IgG trough levels, more infections/year and antibiotic courses/year were potential univariate risk factors. After multivariable analysis (Electronic Supplementary Table 4), we found that the median % of CD4 effector/memory cells was a significant risk factor for increased ILD scores (Figure 5, p = 0.03). Median % of CD4 effector/memory cells >67.6% could identify patients with ILD scores ≥13 with 83.3% sensitivity and 68.2% specificity.

ILD progression was defined as an ILD score increase of ≥1 point/year during follow-up. ILD progression was reported in 7 CVID patients and 1 IgSD/SPAD patient. Univariable and multivariable risk factor analysis did not identify risk factors for ILD progression.

Figure 5: High CD4 effector/memory fractions (>67.7%) in peripheral blood are a risk factor for interstitial lung disease (ILD) scores ≥13 in PAD patients. *A spaghetti plot of ILD scores in the remaining PAD patients showed that patients with CD4+ effector/memory fractions 67.6% had ILD scores 13 more frequently.*



DISCUSSION

This is our third retrospective observational study that quantifies AD and ILD development in PAD patients. Previously we only included CVID and XLA patients. In the current study we included all types of PAD patients who were treated with IRT, with an extended follow-up and a multivariable approach.[7,27] We found that age at baseline ≥40 years and median B cell counts ≤205 were sensitive and

specific predictive factors for increased AD scores. Furthermore, increased AD scores were related to poorer FEV1 and chronic cough. The presence of ≥2.5 infections per year and increased AD scores at inclusion could identify PAD patients at risk for AD progression. Finally, we found that an ILD score of ≥13, as well as a % of CD4 effector/memory cells ≥67.6% were sensitive and specific to predict the presence of radiographically diagnosed GLILD, but we did not identify predictors for progression of GLILD.

Median B cell counts <205 were a predictive factor for increased AD scores. This supports that the reduced function of B cells is an important factor associated with AD development in PAD patients. In accordance with previous research, other predictive markers such as reduced switched memory B cells and IgM levels in the univariate analysis.[13,14,23–26] Age was also associated with increased AD scores; however, we hypothesize that age is not an independent risk factor, but merely an intermediate factor. Older patients probably have more diagnostic delay and longer disease duration with possible undertreatment and are thus potentially more at risk for disease complications, as has previously been described. [23]Increased signs of AD were also related to clinical outcomes such as chronic cough and decreased FEV1 in pulmonary function tests, which further emphasizes the need for clinical measures that will prevent AD and future pulmonary complications in PAD patients.

In this cohort, AD scores did not progress in most, but not all, patients, probably due to adequate IRT. Patients with AD progression despite adequate IRT either had XLA or could be identified by frequent infections, airway disease at baseline and possibly an increased need for prophylactic antibiotics. Frequent infections are the principal pathophysiological mechanism of AD and therefore a risk factor for AD progression. [4–9,12,23] Increased need for prophylactic antibiotics is probably an intermediate identifying factor for patients that either potentially encounter more (subclinical) infections or have extensive bronchiectasis with colonization of bacterial pathogens. Still, prophylactic antibiotics are insufficient to halt AD progression in these patients. This raises the question whether additional therapeutic measures should be taken for patients at risk. We did not find a correlation between AD progression and low baseline IgG trough levels. [27] This might be caused by the fact that we studied all forms of PAD and not only CVID. Alternatively, it could be caused by the fact that IRT dosage and advised target IgG trough levels have increased over the years.[30–32]

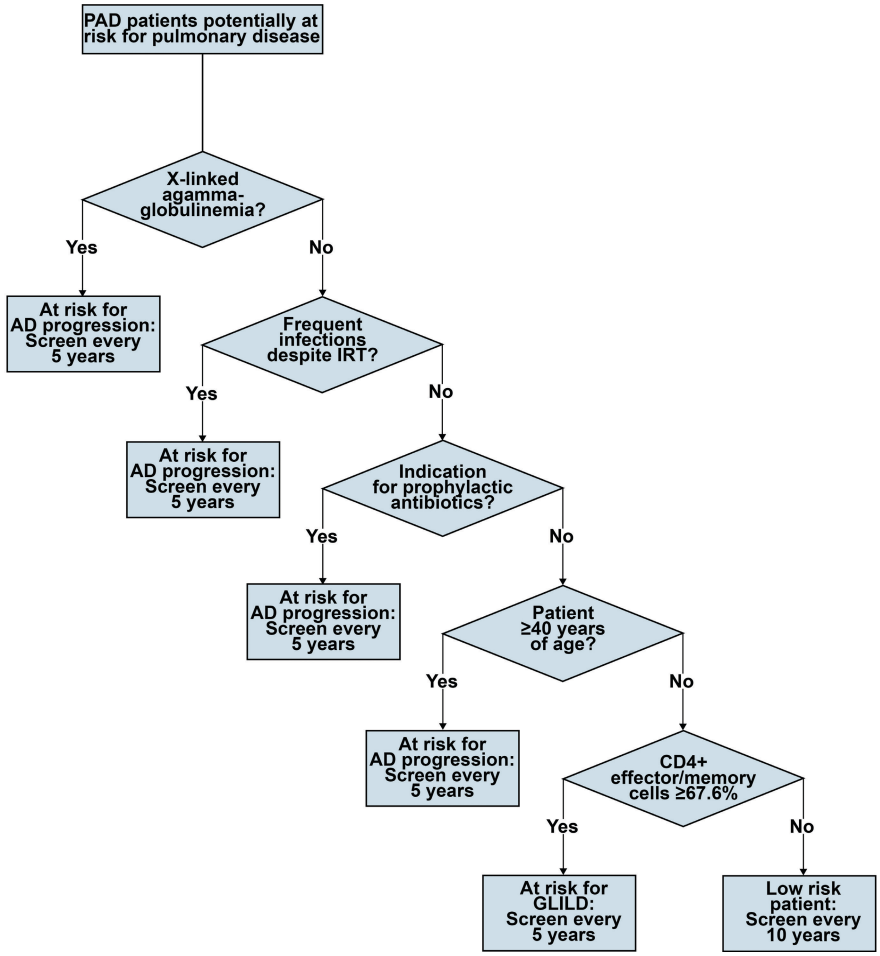
We found that XLA patients had higher AD scores and potentially progressed more often. Diagnostic delay of XLA was is a risk factor for AD progression, similar to the earlier findings of Quinti et al. [23]. Though earlier studies found bronchiectasis to be more common in CVID than XLA, we are the first to show quantified results of severity and progression of AD in XLA compared to other PAD subgroups. [23,29,33,34]

To our knowledge, we are the first to report an ILD score cut-off of ≥13for the Brody-II scoring system that identifies GLILD in PAD patients. Moreover, we found that a high percentage of CD4 effector/memory cells is a risk factor for GLILD. Increased CD4 effector/memory cells correlate with decreased naive CD4 cells and has previously been described as a risk factor for GLILD. (10, 24)(10, 24) This increase may represent chronic immune activation and we speculate that this might be part of the underlying pathophysiological mechanism for GLILD.

Given our results, we advocate that CT screening should be performed at least every 5 years in patients with increased risk for pulmonary complications (Figure 6). Increased screening frequency should be considered in case of (clinical) signs of AD progression, bronchiectasis or recurrent respiratory tract infections. Intensive therapy such as increased IRT dosing, antibiotic prophylaxis, antibiotic treatment of exacerbations and airway clearance techniques taught by chest physiotherapists can be applied to halt AD progression. CT screening frequency could probably be reduced to every 10 years for low-risk patients.

Some limitations of our study should be taken into consideration. Firstly, the retrospective design may have led to selection bias and missing data. Selection bias could have occurred as patients with more severe disease may have undergone more CT scans, pulmonary function tests and blood tests. As a consequence, pulmonary function testing and immunophenotyping might have only been performed in patients with more advanced disease. Second, we used linear stochastic regression imputation to handle missing data. This method might have resulted in an overidentification of interrelationships, since this approach reduces the statistical noise in the dataset. Third, CVID patients were overrepresented in our PAD cohort. We did not detect large differences nor trends in AD scores between CVID, IgSD/SPAD and unPAD patients. Small differences however may have gone unnoticed due to the overrepresentation of CVID patients. Finally, the sample size was not large and we used a data driven approach to define cut-off values. Our study therefore needs independent replication.

Figure 6: Decision tree regarding screening for pulmonary disease in primary antibody deficiency.



AD = Airway Disease, GLILD = granulomatous or lymphocytic interstitial lung disease, IRT = Immunoglobulin replacement therapy, PAD = primary antibody deficiency

In conclusion, we have identified sensitive potential risk factors for the presence of AD in PAD patients. Moreover, we report factors that could identify patients with a greater risk for AD progression despite adequate IRT. We advocate that CT screening frequencies can potentially be reduced for low-risk patients (Figure 6). XLA patients should be studied separately since they have a different disease entity with different risk factors, that potentially requires a different therapeutical- and follow-up approach. To further improve detection and subsequent management of AD in PAD patients, future research should focus on prospective validation of risk factors in all PAD subgroups.

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STATEMENTS AND DECLARATION

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Data collection and analysis were performed by Bas Smits, Sharisa Boland and Marjolein Hol. The first draft of the manuscript was written by Bas Smits and Sharisa Boland and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

SUPPLEMENTARY TABLES

Electronic Supplementary Table 1: *Listing of retrospective gathered variables. Continuous variables were counted as available if they were collected at least once during follow-up. For multivariable risk factor analysis, medians of continuous variables were calculated and used. Missing medians were imputed using bootstrapped (n=100) multiple regression imputation if <40% of the original data was missing.*

FEV 1 = forced expiratory volume in 1 minute, FVC = forced vital capacity, Ig = immunoglobulin, IRT = immunoglobulin replacement therapy

	Availability per diagnosis (missing)					
Parameter	**XLA**	**CVID**	**IgSD/ SPAD**	**unPAD**	**Imputed?**	**Included in risk factor analysis?**
Demographic characteristics	11 (0)	54 (0)	18 (0)	8 (0)	No	Yes
Genetics	11 (0)	21 (33)	6 (12)	6 (2)	No	Yes
Smoking status	11 (0)	54 (0)	17 (1)	8 (0)	Yes	Yes
IRT	11 (0)	54 (0)	18 (0)	8 (0)	No	Yes
Infections	11 (0)	53 (1)	18 (0)	8 (0)	Yes	Yes
Antibiotic Therapy	11 (0)	53 (1)	18 (0)	7 (1)	Yes	Yes
Non-infectious complications	11 (0)	54 (0)	18 (0)	8 (0)	No	Yes
Immunosuppressive therapy	11 (0)	54 (0)	18 (0)	8 (0)	No	Yes
IgG Trough levels	11 (0)	54 (0)	18 (0)	8 (0)	Yes	Yes
IgA/IgM	4 (7)	37 (17)	7 (1)	7 (1)	Yes	Yes
FEV 1	8 (3)	32 (22)	6 (12)	5 (3)	No	No
FVC	8 (3)	32 (22)	5 (13)	5 (3)	No	No
Immunophenotyping	3 (8)	39 (15)	9 (7)	6 (2)	Yes	Yes

Electronic Supplementary Table 2: *Listing of the multivariable risk factor analysis for AD scores ≥7. Potential risk factors form the univariable analysis were included and the variance inflation factor was calculated (model 1). Co-linear variables were then removed from the model and new estimates and p were calculated (model 2).*
AD = airway disease, GLILD = granulomatous or interstitial lung disease, IgM = immunoglobuline M, VIF = variance influencing factor

	Model 1			Model 2		
Variable	**Estimate**	**p value**	**VIF**	**Estimate**	**p value**	**VIF**
Age at baseline	0.04	0.15	3.0	0.04	0.04	1.3
CD19+ counts	-0.01	0.02	2.4	-0.01	0.02	2.3
History of non-infectious complications	-0.45	0.74	5.7	NA	NA	NA
Active non-infectious complications	0.20	0.89	6.2	<0.01	1.0	2.3
GLILD	-0.28	0.81	2.3	-0.11	0.92	1.9
IgM	0.37	0.39	3.2	0.21	0.46	1.4
Diagnostic Delay	0.02	0.60	2.4	0.01	0.72	1.4
CD4+ counts	<0.01	0.72	2.9	<0.01	0.84	1.9
% of CD8 effector/memory cells	<0.01	0.72	3.1	NA	NA	NA
% of switched memory B cells	-0.03	0.54	5.6	NA	NA	NA

Electronic Supplementary Table 3: *Listing of the multivariable risk factor analysis for AD score increase of ≥0.5 points/year. Potential risk factors form the univariable analysis were included and the variance inflation factor was calculated (model 1).*
Co-linear variables were then removed from the model and new estimates and p were calculated (model 2).
AD = airway disease, PAD = primary antibody deficiency, VIF = variance influencing factor

	Model 1			Model 2		
Variable	**Estimate**	**p value**	**VIF**	**Estimate**	**p value**	**VIF**
Age at baseline	0.03	0.29	1.6	0.03	0.23	1.3
History of non-infectious complications	0.93	0.69	9.3	NA	NA	NA
AD ≥7 at baseline	1.87	0.04	1.5	2.74	0.03	1.2
Mean infections per year	1.25	0.63	22.6	1.42	0.01	1.2
Duration of prophylactic antibiotics	2.06	0.15	1.9	2.13	0.08	1.3
Mean courses of antibiotics per year	0.06	0.97	23.2	NA	NA	NA
Active non-infections complications	-0.08	0.97	8.7	0.74	0.42	1.3
PAD duration	0.05	0.29	1.5	0042	0.29	1.1
Smoking	0.04	0.98	1.4	NA	NA	NA
% CD8 effector/memory cells	-0.01	0.87	4.7	NA	NA	NA
CD8 counts	<-0.01	0.27	1.5	<-0.01	0.19	1.2
Median IRT dose	0.89	0.88	1.2	0.17	0.98	1.2

Electronic Supplementary Table 4: *Listing of the multivariable risk factor analysis for ILD scores ≥13. Potential risk factors form the univariable analysis were included and the variance inflation factor was calculated (model 1). Co-linear variables were then removed from the model and new estimates and p were calculated (model 2).*

AD = airway disease, IgG = immunoglobulin G, ILD = interstitial lung disease, IRT = immunoglobulin replacement therapy, PAD = primary antibody deficiency, VIF = variance influencing factor

	Model 1: did not converge	**Model 2**		
Variable	**VIF**	**Estimate**	**p value**	**VIF**
CD19+ counts	10	<-0.01	0.94	1.4
History of non-infectious complications	121	NA	NA	NA
AD ≥7 at baseline	59	0.37	0.76	1.5
Active non-infectious complications	224	NA	NA	NA
% of CD4 effector/memory cells	77	0.08	0.04	1.4
Mean IRT dose	172	NA	NA	NA
Age at baseline	92	<-0.01	0.99	1.2
% of switched memory B cells	3133	NA	NA	NA
PAD diagnosis	129	-1.70	0.99	1.0
Mean IgG trough levels	12	-0.16	0.34	1.1
Mean infections per year	72	0.89	0.23	1.2
% of CD8 effector/memory cells	48	NA	NA	NA

CHAPTER 7

Immunoglobulin Replacement Therapy Versus Antibiotic Prophylaxis as Treatment for Incomplete Primary Antibody Deficiency

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ABSTRACT

Background: Patients with an IgG subclass deficiency (IgSD) or specific polysaccharide antibody deficiency (SPAD) often present with recurrent infections. Previous retrospective studies have shown that prophylactic antibiotics (PA) and immunoglobulin replacement therapy (IRT) can both be effective in preventing these infections; however, this has not been confirmed in a prospective study.

Objective: To compare the efficacy of PA and IRT in a randomized crossover trial.

Methods: A total of 64 patients (55 adults and 9 children) were randomized (2:2) between two treatment arms. Treatment arm A began with 12 months of PA, and treatment arm B began with 12 months of IRT. After a three-month bridging period with cotrimoxazole, the treatment was switched to 12 months of IRT and PA, respectively. The efficacy (measured by the incidence of infections) and proportion of related adverse events in the two arms were compared.

Results: The overall efficacy of the two regimens did not differ ($p = 0.58$, two-sided Wilcoxon signed-rank test). A smaller proportion of patients suffered a related adverse event while using PA (26.8% vs. 60.3%, $p<0.0003$, chi-squared test). Patients with persistent infections while using PA suffered fewer infections per year after switching to IRT (2.63 vs. 0.64, $p < 0.01$).

Conclusion: We found comparable efficacy of IRT and PA in patients with IgSD ± SPAD. Patients with persistent infections during treatment with PA had less infections after switching to IRT.

Clinical Implication: Given the costs and associated side-effects of IRT, it should be reserved for patients with persistent infections despite treatment with PA.

Key words: Primary immunodeficiency, primary antibody deficiency, SPAD, IgSD, prophylactic antibiotics, immunoglobulin replacement therapy, IRT, randomized controlled trial, RCT.

Abbreviations

CVID = common variable immunodeficiency
IgSD = IgG subclass deficiency
IRT = immunoglobulin replacement therapy
IVIG = intravenous immunoglobulins
MBL = mannose-binding lectin
PA = prophylactic antibiotics
PAD = primary antibody deficiency
PID = primary immunodeficiencies
PLS-DA = partial least squares discriminant analysis
SPAD = specific polysaccharide antibody deficiency
URTI/LRTI = upper/lower respiratory tract infections
XLA = X-linked agammaglobulinemia

INTRODUCTION

Low or absent levels of circulating specific antibodies are the hallmark of primary antibody deficiencies (PADs), which cover a spectrum of antibody deficiency syndromes ranging from IgG subclass deficiency (IgSD) and impaired specific polysaccharide antibody production to agammaglobulinemia as the most severe antibody deficiency disease manifestation.[1-3] PADs are the most common type of primary immunodeficiency (PID) with an estimated prevalence ranging from 2.0 to 2.5/10,000 in the United States, excluding patients with IgA deficiency.[4,5] For severe types of PAD such as common variable immunodeficiency (CVID) and X-linked agammaglobulinemia (XLA), evidence-based treatment guidelines involving immunoglobulin replacement therapy (IRT) have been developed.[6-11] In contrast, for the less severe forms of PAD such as IgSD or specific polysaccharide antibody deficiency (SPAD), guidelines are lacking, and both prophylactic antibiotics and IRT are used. Although the costs and possible side effects of these treatments differ significantly, studies that prospectively compare these two regimens have not been published.[8,12,13]

Patients with IgSD and SPAD (in this study defined as patients with incomplete antibody deficiency) present with similar symptoms ranging from asymptomatic to recurrent upper and lower respiratory tract infections (URTI/LRTI).[10,14,15] Although there is no general consensus regarding the treatment of these patients, multiple sources have advocated a step-up approach for the prevention of infections.[8,13,16] As a first-line treatment, additional vaccinations combined with increased vigilance and appropriate antibiotic therapy in the case of bacterial infections can lead to significant clinical improvement. In the absence of improvement, prophylactic antibiotics (PA) are often used to reduce the number of infections.[17-19] Patients with persistent bacterial infections despite PA can be treated with IRT to further reduce the infectious burden.[16,17,20-23]

Open, nonplacebo-controlled studies have shown that both PA and IRT can be effective in patients with IgSD and SPAD. In one study, 22 patients with an IgG2/4 deficiency and recurrent URTIs were treated with cotrimoxazole for 12 months. Twelve of the 22 patients remained symptomatic on antibiotics and were subsequently treated with IRT (intravenous immunoglobulin [IVIG], 400 mg/kg) every 3 weeks. Their mean incidence of URTIs decreased significantly after the introduction of IRT.[12] In another study, 26 patients diagnosed with chronic sinusitis and decreased serum levels of immunoglobulin isotypes, IgSD and/or SPAD were

followed prospectively for one year on prophylactic antibiotics. Nineteen of 26 patients (74%) had a >50% reduction in sinusitis episodes during the 12-month follow-up period.[19] A third retrospective study showed that 22 patients with IgSD and/or SPAD significantly improved after the introduction of IRT with a significant reduction in the number of infections, antibiotic use and hospital admissions per year during the 5-year follow-up period when compared with the year prior to IRT.[22] These findings support the notion that patients with less severe forms of PAD such as IgSD and SPAD who suffer from recurrent RTIs can benefit from either PA or IRT. Recently, a fourth retrospective study was published comparing PA (different types, N=7) and IRT (N=29) in children with SPAD. The authors reported a comparable mean number of infections in patients on PA vs. IRT (2.86, SD: 2.73 vs. 4.44, SD: 4.74) during the 1-year follow-up period; however, 15 patients (23.1%) failed on PA and switched to IRT during the year.[24] Moreover, the mean number of infections did not decrease in patients who received a combination of PA and IRT (N=7).[24] However, the numbers of patients in most of these studies were small, and evidence that one of these treatment modalities is truly superior to the other is lacking because these therapies have not been compared in a prospective randomized study with a larger cohort. As IRT is an expensive therapy for which global demand is increasing, it is important to establish which patients benefit the most from this type of therapy.[25]

We aimed to compare the efficacy and side effects of prophylactic antibiotics vs. intravenous immunoglobulin therapy in patients with IgG subclass deficiency and/or specific polysaccharide antibody deficiency using a randomized, crossover experimental design.

METHODS

Study design

The trial (P06.233/P08.034) was designed as a multicenter, randomized phase IV trial with a crossover design comparing the use of PA vs. IRT among IgSD and SPAD patients with recurrent infections. Block randomization was performed in a 2:2 ratio, dividing the participants between treatment arms A and B. Treatment arm A consisted of 12 months of prophylactic treatment with cotrimoxazole followed by a 3-month bridging period in which cotrimoxazole was also used as a prophylaxis, followed by 12 months of intravenous immunoglobulins (IVIG) (Figure 1). Treatment arm B began with a 12-month period of IVIG followed by a 3-month washout period (during which antibiotics were given) and a 12-month period of

prophylactic cotrimoxazole. A minimum of 45 participants was required for the assessment of the primary endpoint (the number of infections per patient per year). The Institutional Review Board (IRB) of Leiden University Medical Center operated as the central IRB that reviewed and approved this study. All participants signed written informed consent prior to participation in the study.

Objectives and endpoints

The primary objective was to measure the difference in the number of infections per patient per year between the two treatment modalities. The secondary predefined endpoints were a reduction in the total duration of infections, a reduction in severe infections, fewer periods of fever, fewer hospital admissions and days absent from school/work due to infections, and improvement in the Karnofsky performance skill index during the study. The number and duration of infections were reported by the treating physician and infections were classified from mild to severe using predefined definitions (Table S1).

The secondary objective was to assess side effects and tolerability through the evaluation of laboratory variables and (serious) adverse events. The intensity of Adverse Events (AEs) was classified as mild, moderate or severe (Table S2). Moreover, as an exploratory objective, this study evaluated possible discriminative variables that could identify patients who benefit from IRT.

Eligibility

This study included patients >5 years of age with an established diagnosis of IgG_1, IgG_2 and/or IgG_3 subclass and/or anti-polysaccharide antibody deficiency from eight (tertiary) hospitals in the Netherlands. IgSD was defined as IgG_1, IgG_2 and/or IgG_3 serum levels below the age-adjusted lower reference range, which was determined on two occasions. SPAD was defined as an insufficient increase in anti-pneumococcal antibody formation for >50% of the measured serotypes after vaccination with a 23-valent polysaccharide pneumococcal vaccine (Pneumo23). A sufficient increase in anti-pneumococcal antibody formation was defined as a 4-fold rise in antibody titers measured 3-5 weeks after vaccination or a serum titer > 1 mg/ml (or >20 IU/L) after vaccination. In patients with a previous pneumococcal conjugate vaccination, only nonconjugate serotypes were considered. The diagnosis of SPAD was excluded for patients with a protective anti-pneumococcal antibody titer upon vaccination. Other inclusion criteria were a total serum IgG level > 4 g/L and at least 2 physician-diagnosed infections in the 6 months prior to inclusion in the study.

Participants were excluded if they were treated with any other investigational drug within a week before entry into the study, if they had a history of allergic reactions to either immunoglobulin treatment or cotrimoxazole, if they had a progressive terminal disease or active systemic disease, if they were pregnant, or if they were known to have renal insufficiency.

Treatment description

IVIG was infused every three weeks dosed at 600 mg/kg for patients ≥18 years old; younger patients were given 800 mg/kg every three weeks. Nanogam® was used as the intravenous immunoglobulin product and was supplied by Sanquin Plasma Products BV, Amsterdam, the Netherlands.[26] Cotrimoxazole was chosen to be used as an antibiotic prophylaxis, based on data from previous retrospective cohorts and national guidelines. Cotrimoxazole has shown to be effective against the most prevalent bacteria that cause respiratory tract infections and has also been confirmed to be effective as prophylaxis in both PAD and non-PAD patients.[12,27-30] It was dosed once daily at 160 mg trimethoprim/800 mg sulfamethoxazole for participants ≥ 12 years old or above 40 kg body weight, whereas younger participants were given 4 mg trimethoprim/20 mg sulfamethoxazole per kg bodyweight. If patients had a known intolerance for cotrimoxazole or if it was not tolerated during the trial, azithromycin was given three days per week dosed at 500 mg per day for participants >18 years old or 10 mg per kg body weight for younger participants. Patients who developed three or more respiratory tract infections during one study period were switched to a combined regimen of IRT and PA, dosed as previously described, after the third infection.

Laboratory variables

As secondary markers of efficacy, we performed lymphocyte phenotyping (performed at the Medical Immunology and the Immunodiagnostic Laboratory of the Erasmus MC),[31] mannose-binding lectin (MBL) genotyping and determined the MBL concentration (performed at the Laboratory of Blood Cell Research and Immunochemistry of Sanquin diagnostics, BV) at the start of the study. Moreover, leukocyte counts, hemoglobin levels, hematocrit, platelet counts, and potassium, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactic dehydrogenase, serum creatinine and antibody levels were measured at the start of the study and once every 3 months thereafter.

Statistics

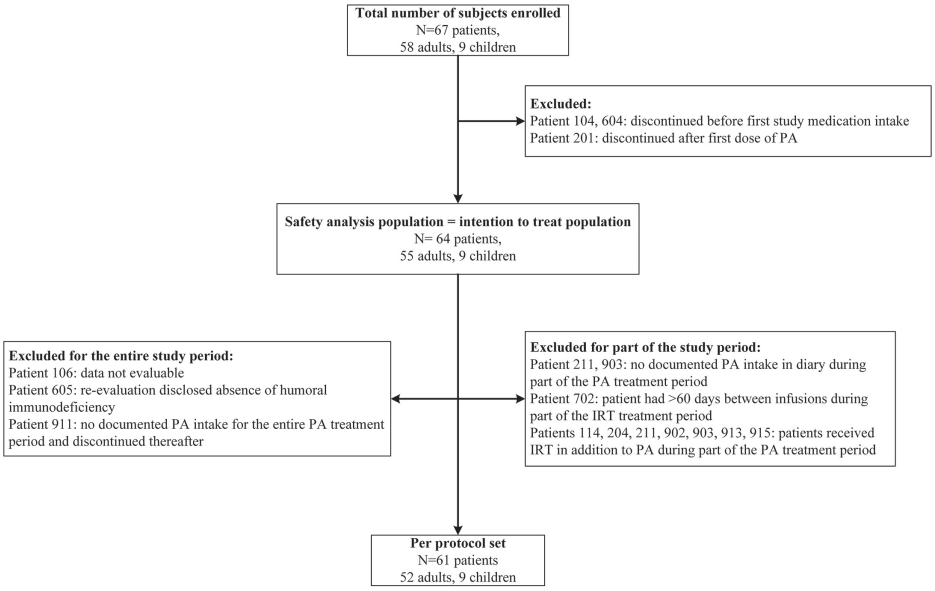
A minimum sample size of N=35 was calculated to prove noninferiority with a standard deviation of 2 and a noninferiority limit of 1.5 infections per year (α= 0.05, 90% power) between the two treatment arms, however since a larger sample size was reached, this study was powered to prove noninferiority within a limit of 1.15 infections per patient per year between the two treatment arms. Baseline characteristics were summarized by age group, and study outcomes were summarized by treatment arm with participants classified according to the intention to treat (ITT) principle. For the mean number of infections per patient per year, the standard deviation was estimated and the difference was analyzed with a two-sided Mann-Whitney U test. For other endpoints, categorical outcomes were described using proportions and compared between the arms using a Chi-squared test. Outcomes were corrected for multiple comparisons using the Holms-Bonferroni approach. A Cox proportional hazard model was used to compare the time to first infection in both treatment groups. Moreover, a per-patient analysis was performed using the reduction in infection rates to define a subgroup of patients who might benefit from IRT; a reduction of at least 1 infection per year was set as the cutoff. Differences between baseline variables in patients with and without persistent infections were further visualized by fitting a hierarchal clustering model and by a supervised clustering method (partial least squares discriminant analysis, PLS-DA) using a one-component model. R Studio version 1.2.5019 and SAS version 9.4 were used to analyze the data.

RESULTS

Enrolled and randomized participants

A total of 67 patients from 8 centers were enrolled in the study (Figure 1) of whom 58 were adults and 9 were children. Three participants discontinued before randomization (withdrawal of informed consent), and hence a total of 64 patients was available for the intention to treat analysis. Data from three adult participants were excluded from the per-protocol analysis for the following reasons: insufficient data (N=1), misdiagnosis (no PAD, N=1) and no documented intake of study medication (N=1). Furthermore, data from 8 participants were partially censored for the per-protocol analysis because they were treated with IVIG in addition to antibiotic prophylaxis (3 during IRT treatment and 5 during PA treatment) or had no documented intake of antibiotics for part of the study period.

Figure 1. Inclusion tree of the study; *67 patients were initially enrolled of which 3 discontinued before the start of the study. A total of 55 adults and 9 children were included in the intention to treat population. For the per-protocol analysis, 52 adults and 9 children were included of which data from 8 patients was partially censored because they were treated with IVIG in addition to antibiotic prophylaxis or had no documented intake of antibiotics for part of the study period.*



Baseline characteristics

Baseline characteristics and the results of blood counts, immunoglobulin levels, B cell maturation markers, specific antibody titers and MBL activity are presented in Table 1. After correcting for multiple comparisons, only the IgG2 levels were found to be significantly lower in children compared with those in adults ($p < 0.0001$). This was anticipated because the normal ranges for IgG2 are lower for children than for adults;[32] children and adults were pooled in further analyses. The mean IgG trough level of participants receiving IRT was 11.77 g/L (95% CI: 10.8–12.7). Mean levels of IgM and IgA did not differ significantly among the children and adults included in this cohort.

Table 1. Baseline characteristics of the adults and children analyzed in the intention to treat analysis. *Mean values were compared with a Mann-Whitney U test. *Indicates significance after correction for multiple comparisons using the Holms-Bonferroni method.*

	Adults	Children	p-value
Sex (n):			
- **Male**	21	7	
- **Female**	34	2	
Age (median)	50	8	
Diagnosis (n):			
- **IgSD**	34	2	
- **SPAD**	3	1	
- **Both**	18	6	
Hemoglobin (mmol/L)	8.66	8.27	0.133
Leukocytes (10^9/L)	7.80	5.06	0.023
Thrombocytes (10^9/L)	259	265	0.858
Neutrophils (10^9/L)	5.18	9.22	0.015
Eosinophils (10^9/L)	0.13	0.29	0.206
Lymphocytes (10^9/L)	2.07	2.03	0.629
Monocytes (10^9/L)	0.54	0.31	0.009
IgG:			
- **Total IgG l**	7.76	7.14	0.766
- **IgG1**	5.12	6.05	0.143
- **IgG2**	1.90	0.62	<0.0001*
- **IgG3**	0.27	0.33	0.638
- **IgG4**	0.27	0.23	0.656
IgA	1.39	0.69	0.017
IgM	1.35	0.65	0.169
IgE (g/L)	88.7	120	0.150
CD19 (%)	11.4	11.3	0.492
CD27 (%)	33.7	17.6	0.028
CD21 (%)	85.8	88.7	0.467
CH50 (%)	107.6	102.3	0.507
AP50 (%)	94.3	66.5	0.265
MBL Expression (mg/mL)	0.38	3.20	0.081
Low MBL Expression (<0.8)	21/55	2/9	
MBL Polymorphism in Exon 1	15/55	3/9	
MBL Polymorphism in Promotor	42/55	6/9	
Low MBL Expression + Exon 1 Polymorphism	1/55	0/9	
Low MBL Expression + Promoter Polymorphism	2/55	0/9	
Low MBL Expression + Exon 1 & Promoter Polymorphisms	13/55	2/9	

IgSD = Immunoglobulin G subclass deficiency, SPAD = specific polysaccharide antibody deficiency, Ig= immunoglobulin, CD = cluster of differentiation, and MBL = mannose-binding lectin.

Primary outcome: infection prevention

A total of 64 participants were evaluable for the primary endpoint analysis (IRT N=58, PA N=56). A total of 19,618 treatment days were recorded for the IRT group vs. 20,256 for the PA group. There was no proof of statistically significant superiority of either treatment arm with a mean of 1.76 (SD: 1.92) infections per patient per year in the IRT arm vs. 1.55 (SD: 1.94) in the PA arm (Table 2). In the IRT group, 76.4% of the infections were RTIs vs. 73.8% of those in the PA group. Of all the participants, 70.7% in the IRT arm and 51.8% in the PA arm had at least one infection during the intervention period. However, this was not found to be significant in a Cox proportional hazards model (Figure 2). To evaluate the therapeutic potential of a combined therapy with both IRT and PA, the censored data of the 7 participants receiving a combination therapy was compared with the study population; however, no significant reduction in the incidence of infection was found.

Table 2: Primary outcome measures in the intention to treat (ITT) and the per-protocol set (PPS). *Chi-squared tests or Mann-Whitney U tests were used accordingly to calculate the p-values; no significant differences were found.*

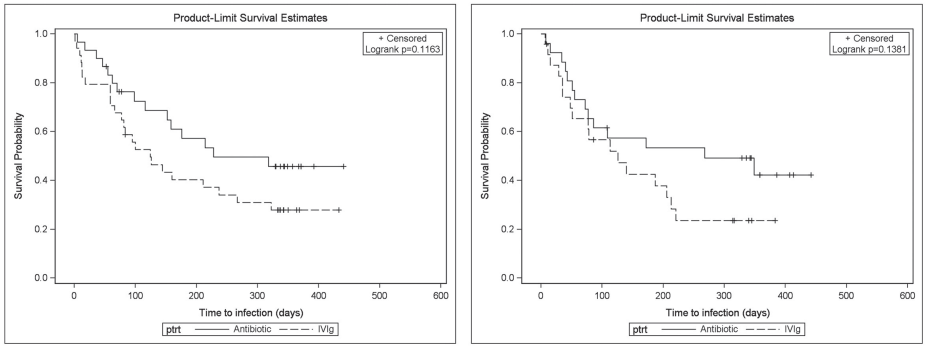
		ITT			PPS		
		IRT	**PA**	**p-value**	**IRT**	**PA**	**p-value**
Number of patients	All	58	56		57	54	
	Children	7	9		7	9	
	Adults	51	47		50	45	
Number of treatment days	Sum	19618	20256		19228	19495	
	Mean (SD)	338.2 (85.3)	361.7 (79.9)		337.3 (90.5)	361.0 (73.8)	
Number of infections	All	89	84		88	82	
	Children	4	10		4	10	
	Adults	85	74		84	72	
Duration (n)							
	Acute	84	75		83	73	
	Chronic	5	9		5	9	
Intensity (n)							
	Mild	48	49		48	47	
	Moderate	35	30		34	30	
	Severe	6	5		6	5	
Patients suffering ≥1 infection (%)		41 (70.7%)	29 (51.8%)	0.09	40 (70.2%)	29 (53.7%)	0.15
Infections per patient per year (SD)		1.76 (1.92)	1.55 (1.94)	0.56	1.77 (1.94)	1.57 (1.90)	0.58

Table 2: Continued from previous page.

	ITT			PPS		
	IRT	**PA**	**p-value**	**IRT**	**PA**	**p-value**
Severe infections per patient per year (SD)	0.11 (0.60)	0.09 (0.36)	0.83	0.11 (0.60)	0.09 (0.36)	0.83
Days of infection per patient year (SD)	24.6 (30.0)	23.1 (42.0)	0.83	24.6 (30.3)	23.7 (42.4)	0.85

ITT = intention to treat, PPS = per-protocol set, IRT = immunoglobulin replacement therapy, PA = prophylactic antibiotics, and SD = standard deviation.

Figure 2. Kaplan-Meier curves for the time to first infection for Treatment period 1 (left) and Treatment period 2 (right). *There was a trend towards a longer time to first infection for the antibiotics group, but this was not statistically significant (p = 0.116 and p = 0.138, respectively, for periods 1 and 2).*



Period 1:
Log-rank test p-value: 0.116
Antibiotics: n = 30 IRT: n = 34

Period 2:
Log-rank test p-value: 0.138
Antibiotics: n = 26 IRT: n = 24

Secondary outcome parameters

To further quantify the effect of the treatments in both study arms, secondary outcome measures were compared. The mean total duration of infections per year and the number of severe infections (Table 2), days off school/work, febrile episodes and hospitalization admissions were analyzed (Table 3); no significant differences were found. The mean total duration of infections per patient per year was 24.6 d (SD: 30.0 d) in the IRT group vs 23.1 d (SD: 42.0 d) in the PA group. The mean number of severe infections per patient per year was 0.11 (SD: 0.60) in the IRT group vs 0.09 (SD: 0.36) in the PA group. Respiratory tract infections were the most commonly reported infection (68% of all infections) and were equally reported in both treatment arms.

Table 3: Secondary outcome measures in the intention to treat (ITT) and the per-protocol set (PPS). *Chi-squared tests or Mann-Whitney U tests were used accordingly to calculate p-values.*

	ITT			PPS		
	IRT	**PA**	**p-value**	**IRT**	**PA**	**p-value**
Total number of patients	58	56		57	54	
Total number of infections	89	84		88	82	
Days off work or school						
Total number of days off work/school	120	65		90	65	
Patients (%) with at least one day off work/school	7 (12.1%)	7 (12.5%)	0.94	6 (10.5%)	7 (13.0%)	0.61
Number of days off work/school per patient per year (mean ± SD)	2.3 (9.3)	1.2 (3.4)	0.41	1.6 (7.9)	1.2 (3.4)	0.73
Fever						
Total number of fever events	21	24		21	22	
Patients (%) experiencing at least one fever event	12 (20.7%)	13 (23.2%)	0.71	12 (21.1%)	13 (24.1%)	0.66
Number of fever events per patient per year (mean ± SD)	0.4 (1.2)	0.5 (1.0)	0.63	0.5 (1.2)	0.4 (1.0)	0.64
Hospitalization due to infection						
Total number of hospital admissions due to infection	14	10		13	10	
Total number of days in the hospital due to infection	112	81		89	81	
Patients (%) with at least one hospital admission	6 (10.3%)	7 (12.5%)	0.65	5 (8.8%)	7 (13.0%)	0.37
Number of hospital admissions per patient per year	0.30 (1.10)	0.19 (0.58)	0.51	0.28 (1.10)	0.20 (0.59)	0.64
Average number of days in the hospital per person year (mean ± SD)	2.80 (10.11)	1.49 (4.22)	0.37	2.32 (9.51)	1.55 (4.29)	0.59

ITT = intention to treat, PPS = per protocol set, IRT = immunoglobulin replacement therapy, and PA = prophylactic antibiotics.

Predictors of benefit from IRT

To evaluate the possibility of a subgroup of patients who benefited from IRT over PA, a per-patient analysis was performed. The reported infection rates were analyzed and the reductions in infection rates were calculated. In this analysis, we identified 11/58 patients with a reduction of at least 1 infection per year when treated with IRT instead of antibiotics (monotherapy, as prescribed in the treatment protocol). To further characterize these participants, a PLS-DA was performed on all baseline characteristics available. This analysis yielded no markers that could identify this subgroup of patients who benefited from IRT over PA. Next, we analyzed the number of infections during treatment with PA as a separate variable, and found that patients who suffered ≥2 infections during treatment with PA generally had a beneficial response when switched to IRT. Specifically,

the occurrence of ≥2 infections despite the use of PA monotherapy identified participants who subsequently improved following a switch to IRT monotherapy with 80% (CI: 44.4–97.5) sensitivity and 80.6% specificity (CI: 64.0–91.8). Moreover, most of these infections (86%) were RTIs. In this subgroup of patients with persistent infections despite PA, there was a significant ($p<0.01$) reduction in the mean number of RTIs from 2.63 (SD: 2.20) RTIs per patient per year to 0.64 (SD: 0.81) for patients treated with IRT.

Table 4: *Total numbers and proportions of (serious) adverse events among the patients in the antibiotics and IRT groups. Chi-squared tests were used to calculate the p-values. *Indicates significance after correction for multiple comparisons using the Holms-Bonferroni method. The total number of events and the number of related serious events were lower in the antibiotics group.*

	Statistic	PA	IRT	p-value
Any adverse events	Number of events % (n/N)	93 66.1% (37/56)	270 79.3% (46/58)	0.1149
Any related adverse events	Number of events % (n/N)	34 26.8% (15/56)	184 60.3% (35/58)	0.0003*
Any serious adverse events (total)	Number of events % (n/N)	13 12.5% (7/56)	26 19.0% (11/58)	0.3437
Serious adverse events (AE-related)	Number of events % (n/N)	1 1.8 (1/56)	14 13.8% (8/58)	0.0181*
Serious adverse event (infection-related)	Number of events % (n/N)	12 12.5% (7/56)	12 10.3% (6/58)	0.7128

PA = prophylactic antibiotics, IRT = immunoglobulin replacement therapy, and AE = adverse event.

Tolerability of medication in both treatment arms

The key secondary objective of this study was to compare the tolerability of PA and IRT by analyzing the proportion of patients who suffered any adverse events (AE) that were possibly related to treatment. A total of 64 participants received at least one dose of study medication and had reports of safety measurements. Overall, 37 subjects (66.1%) experienced at least one AE during PA treatment, while there were only 46 such subjects (79.3%) during IRT. The most commonly reported AEs for both treatment arms were diarrhea, nausea, fatigue, pyrexia, headache and rash for both the total number of AEs and the AEs possibly related to treatment (Table S3). In total, 40 participants (62.5%) experienced at least one AE that was at least potentially related to the study drug: 15 in the PA arm (26.8%) and 35 in the IRT arm (60.3%, $p = 0.0003$, Table 4). Patients treated with IRT experienced headache more often than patients treated with PA (36.2% vs. 1.8%, $p<0.0001$). The intensity of the adverse events that were possibly related to treatment was lower in the PA arm than in the IRT arm; 25% of patients in the PA arm had AEs of mild intensity, 1.8% of patients had AEs of moderate intensity and 0% of patients

had AEs of severe intensity vs. 53.4%, 19.0% and 3.4% in the IRT arm, respectively (p = 0.002). Most (60%) of the AEs reported in the IRT group were reported during the first four infusions. Moreover, 8 patients (5 in Treatment Arm A, 3 in Treatment Arm B) were treated with azithromycin, due to known or acquired intolerability of co-trimoxazole

Overall, 13 serious adverse events (SAE) were reported by 7 participants (12.5%) during PA and 26 SAEs were reported by 11 participants (19%) during IRT. Of these SAEs, 24 originated from infection-related events (N=12 during PA and N=12 during IRT). One of the SAEs was identified as probably related to IRT. The patient was admitted to the hospital with fever, lymphadenopathy, myalgia, arthralgia, malaise and leucopenia of unknown origin from which the patient recovered after 36 days, during which IRT was continued.

DISCUSSION

This multicenter, randomized, controlled crossover trial is the first to analyze the efficacy of PA versus IRT in patients with IgSD and/or SPAD. Overall, our data did not demonstrate a significant difference in infection-related parameters between the two regimens, and PA was better tolerated than IRT. Moreover, the Kaplan-Meier models do suggest a trend towards longer infection free survival among patients in the PA group. Increasing the sample size might have powered the study enough to confirm this finding. However, the subgroup of patients with persistent infections despite treatment with PA had significantly fewer infections when treated with IRT.

Overall, the data from this study are generalizable to other patients with IgSD ± SPAD and recurrent infections, as trial eligibility criteria allowed the participation by both children and adults with IgSD, SPAD or both, and children and adults from both disease categories were included. Unfortunately, only four patients were included that suffered specifically from SPAD, making this study less generalizable for this patient group. However, the mean infection rates reported were comparable to those reported in two earlier studies, one in children with SPAD[24] and one in adults with IgSD and SPAD.[22] The current study design, a randomized, controlled crossover trial, was selected as the optimal design to show differences in the efficacy of prophylactic treatment in a relatively rare disease where the cohort sizes are expected to be small.[33] Both treatment arms covered 12 months to avoid seasonal differences in infections. To ensure that successive infections

were equally weighted in the analysis, the duration and severity of infections were included in the analyses. Moreover, good compliance was achieved as 53/64 patients completely adhered to the protocol, resulting in sample sizes sufficient to perform a per-protocol analysis.

The crossover design of the study allowed further study of the potential benefit of IRT over PA. Using subgroup analysis, we were able to define a subgroup of patients with persistent infections despite PA treatment who benefited from switching to IRT. This group had ≥2 infections per year despite treatment with PA and showed a statistically significant ($p<0.01$) and clinically relevant reduction in infections after switching to IRT. Apart from the persistence of infections while using PA, we found no other (laboratory) parameters identifying patients who would benefit from IRT over PA. Joud Hajjar et al. showed that patients with persistent infections had lower IgG titers than patients who were responsive to PA or IRT; however, we could not replicate this result in our cohort.[24] We also analyzed MBL, as MBL deficiency was hypothesized to be only clinically relevant in patients with concurrent immune deficiencies; however, we found no clinically relevant effect of MBL deficiency on infections even in combination with the antibody deficiencies studied in our protocol. Our data suggest that an empirical step-up approach, beginning with antibiotic prophylaxis and switching to IRT monotherapy in response to persistent infections, is valid for the treatment of IgSD ± SPAD. IRT reduced the infectious burden in patients on PA who suffered from two or more infections in 56% of the cases.

The tolerability analyses of this study showed that more AEs that were possibly related to treatment occurred in the IRT group. However, the majority of these AEs occurred during the initial IRT infusions, and most of these AEs were transient, mild and were well-known adverse reactions to IVIG. It is known that the number of AEs decreases over time, as dosage and infusion rate are adjusted based on their occurrence.[34] Moreover, the risk of AEs differed in the two study arms, as patients with a known intolerability to cotrimoxazole or who experienced side effects from antibiotics during the study were switched to an alternative drug (azithromycin instead of cotrimoxazole), whereas the protocol for patients on IRT did not include an option for a different type of IRT in the study. Overall, 8 patients were treated with azithromycin instead of cotrimoxazole, which may have caused a reduction in AEs within the PA group.

The limitations of this study are similar to those of other smaller randomized studies of rare diseases, which are often constrained by recruitment challenges. First, during recruitment, several study candidates chose not to participate in the study, as they preferred to adhere to the treatment they currently received. It is not clear how many patients received no treatment, PA, or IRT, and whether this may have introduced selection bias. However, the study still had sufficient power to show that there was no significant difference in the efficacy between PA and IRT and was able to detect noninferiority with a limit of 1.2 infections per patient per year. Unfortunately, the study was not sufficiently powered to pick up a significant difference in infection free survival time between the two study arms or to draw any concrete conclusions for patients affected by SPAD specifically. Second, the protocol described relatively high substitution doses of IVIG, which were not always achieved. However, it is known that the dose of IVIG is not a good measure of effectivity and that IgG trough levels in combination with a reduced frequency of sinopulmonary infections are recommended instead.[9,11] In CVID, trough levels of 6-8 g/L have been advised for the optimal prevention of infections; this was achieved in all patients who received IRT in this study.[35,36] Third, this study did not analyze the effect of PA on antibiotic resistance. However, a recent systematic review that studied the use of PA in patients with chronic obstructive pulmonary disease found no direct evidence for an increase in antibiotic resistance.[37] A recent study in CVID patients confirmed this for azithromycin in PID patients during a three-year follow-up period.[38] However, these findings might not be generalizable and the outcomes for our study population could differ. Antibiotic resistance is a growing problem that could represent a serious threat particularly in immunodeficient patients who are more susceptible to infections.[39,40] Finally, the results of this study might not be replicable in a non-trial setting due to a possible difference in patient compliance between the two regimens. Non-compliance for oral medication has been extensively studied in the past and up to 50% of patients have been reported as non-compliant with medication.[41] Due to its nature IRT could counteract some of the hurdles patients experience in complying to prophylactic treatment.[42]

In conclusion, we found that overall, prophylactic antibiotics and IRT were equally efficient in preventing infections in a cohort of patients with IgSD ± SPAD. However, a subgroup of patients with persistent infections during treatment with PA showed a significant reduction in infections after switching to IRT. Future research should focus on identifying biomarkers to better define this group.

Acknowledgments

We wish to acknowledge the assistance provided by R. Albers-Strik, J. van Esch and M. van Aartrijk in patient recruitment and data collection, which was greatly appreciated. Moreover, we would like to thank G. Rijkers for measuring the specific polysaccharide antibodies and Prof. P.J.J. Hooykaas and M. van der Burg for measuring the B-cell subsets in the blood samples of our patients. We thank Sanquin Home Service for making it possible for patients to have IVIG infusions at home and for collecting blood samples.

Author Contributions

I.K.B., E.D.V., I.T.B., R.B., M.V.D., J.V.D., P.E., M.V.D.F., P.V.H., C.N., A.R., E.S., A.S., T.K., and J.M. were involved in data collection; I.K.B., E.D.V., J.V.D, E.S. and T.K. designed the research; B.S., I.K.B. and J.M. analyzed results and made the figures and B.S., I.K.B. and J.M. wrote the paper

Conflict of Interest Disclosures

JvM served on an advisory board for Takeda.

IKB is working at Sanquin Plasma Products, Market Authorization Holder of Nanogam

EdV received unrestricted research grants from Sanquin, CSLBehring and Shire/ Takeda, and served on an advisory board for CSLBehring and a patient awareness panel as well as professional education program sponsored by Sanquin.

MvdF received funding from CSL Behring and Takeda.

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SUPPLEMENTAL TABLES

Table S1. Examples of Infections Classified into Mild, Moderate and Severe[a]

Mild	Moderate[a]	Severe[a]
Nasal discharge	Sinusitis[b]	Pneumonia
Flu-like illness	Bronchitis	Pleurisy/pleuritis
Conjunctivitis	Pharyngitis	Acute sinusitis[b]
Diarrhoea	Laryngitis	Acute abscesses
Vaginal infection	Otitis	Sepsis
Oral Candida	Fever[c]	Acute Osteomyelitis
Afebrile enteritis	Urinary tract infection	Meningitis

[a] Moderate infections did not require bed rest or absence from school or work.
Severe infections always required bed rest or hospitalisation and absence from school or work.
[b] Moderate sinus-pressure, headaches
Severe sinusitis-with fever, positive x-rays and necessity of antibiotics
[c] Fever of unknown origin lasting no more than 3 days

Table S2. Adverse Event intensity and severity grading criteria

Adverse Event grading criteria	
Intensity	**Seriousness**
Mild: The patient is aware of the sign/symptom, but it does not interfere with his/her usual activities and/or is of no clinical consequence.	**Severe (SAE**): A serious adverse event is any untoward medical occurrence or effect that at any dose: - results in death or is life threatening; - requires hospitalisation or prolongation of hospitalisation; - results in persistent or significant disability or incapacity; - is a congenital anomaly or birth defect; - is a new event of the trial likely to affect the safety of the subjects
Moderate: The adverse event interferes with usual activities of the patient, or is of some clinical consequence.	**Non-serious:** All events that cannot be graded as serious.
Severe: The patient is unable to carry out his/her usual activities and if applicable unable to go to school, or the adverse event is of definite clinical consequence.	

Table S3. Treatment Emergent Adverse Events: Incidence by System Organ Class and Preferred Term

	Antibiotic Period 1+2		IVIg Period 1+2	
System Organ Class Preferred Term	**Events**	**%(n/N)**	**Events**	**%(n/N)**
OVERALL	93	66.1% (37/56)	270	79.3% (46/58)
Blood And Lymphatic System Disorders	0	-	3	5.2% (3/58)
Anaemia	0	-	2	3.4% (2/58)
Leukopenia	0	-	1	1.7% (1/58)
Cardiac Disorders	0	-	2	3.4% (2/58)
Palpitations	0	-	2	3.4% (2/58)
Ear And Labyrinth Disorders	2	3.6% (2/56)	0	-
Cerumen Impaction	1	1.8% (1/56)	0	-
Tinnitus	1	1.8% (1/56)	0	-
Eye Disorders	1	1.8% (1/56)	0	-
Cataract	1	1.8% (1/56)	0	-
Gastrointestinal Disorders	27	26.8% (15/56)	28	24.1% (14/58)
Abdominal Discomfort	1	1.8% (1/56)	1	1.7% (1/58)
Abdominal Pain	3	5.4% (3/56)	2	3.4% (2/58)
Abdominal Pain Upper	2	3.6% (2/56)	0	-
Constipation	2	3.6% (2/56)	1	1.7% (1/58)
Diarrhoea	8	10.7% (6/56)	8	6.9% (4/58)
Diverticulum	0	-	1	1.7% (1/58)
Dyspepsia	2	3.6% (2/56)	0	-
Gastric Disorder	1	1.8% (1/56)	0	-
Gastrointestinal Motility Disorder	1	1.8% (1/56)	1	1.7% (1/58)
Haematochezia	0	-	1	1.7% (1/58)
Haemorrhoids	0	-	1	1.7% (1/58)
Nausea	6	8.9% (5/56)	11	12.1% (7/58)
Oesophagitis	1	1.8% (1/56)	0	-
Vomiting	0	-	1	1.7% (1/58)
General Disorders And Administration Site Conditions	11	8.9% (5/56)	76	48.3% (28/58)
Asthenia	0	-	1	1.7% (1/58)
Chest Discomfort	0	-	1	1.7% (1/58)
Chest Pain	0	-	3	5.2% (3/58)
Chills	0	-	5	5.2% (3/58)
Discomfort	0	-	5	3.4% (2/58)
Fatigue	4	5.4% (3/56)	15	10.3% (6/58)
Feeling Cold	0	-	5	3.4% (2/58)

Table S3. Continued from previous page.

System Organ Class Preferred Term	Antibiotic Period 1+2		IVIg Period 1+2	
	Events	**%(n/N)**	**Events**	**%(n/N)**
Gait Disturbance	0	-	1	1.7% (1/58)
Influenza Like Illness	1	1.8% (1/56)	4	5.2% (3/58)
Infusion Site Erythema	0	-	2	3.4% (2/58)
Infusion Site Extravasation	0	-	2	1.7% (1/58)
Infusion Site Inflammation	0	-	1	1.7% (1/58)
Infusion Site Phlebitis	0	-	3	1.7% (1/58)
Infusion Site Rash	0	-	1	1.7% (1/58)
Malaise	0	-	4	6.9% (4/58)
Oedema Peripheral	0	-	1	1.7% (1/58)
Pyrexia	6	5.4% (3/56)	22	15.5% (9/58)
Immune System Disorders	2	3.6% (2/56)	1	1.7% (1/58)
Drug Hypersensitivity	1	1.8% (1/56)	0	-
Seasonal Allergy	1	1.8% (1/56)	1	1.7% (1/58)
Infections And Infestations	7	10.7% (6/56)	8	10.3% (6/58)
Body Tinea	1	1.8% (1/56)	0	-
Folliculitis	2	1.8% (1/56)	0	-
Fungal Skin Infection	0	-	2	3.4% (2/58)
Herpes Simplex	0	-	1	1.7% (1/58)
Lymphangitis	0	-	1	1.7% (1/58)
Nasopharyngitis	1	1.8% (1/56)	4	6.9% (4/58)
Rhinitis	1	1.8% (1/56)	0	-
Vulvovaginal Mycotic Infection	2	3.6% (2/56)	0	-
Injury, Poisoning And Procedural Complications	4	5.4% (3/56)	7	8.6% (5/58)
Contusion	0	-	1	1.7% (1/58)
Corneal Abrasion	1	1.8% (1/56)	0	-
Fall	1	1.8% (1/56)	1	1.7% (1/58)
Hand Fracture	0	-	1	1.7% (1/58)
Incorrect Route Of Drug Administration	0	-	1	1.7% (1/58)
Joint Dislocation	0	-	1	1.7% (1/58)
Joint Injury	0	-	1	1.7% (1/58)
Limb Injury	0	-	1	1.7% (1/58)
Post Procedural Haematoma	1	1.8% (1/56)	0	-
Rib Fracture	1	1.8% (1/56)	0	-
Investigations	3	5.4% (3/56)	3	3.4% (2/58)
Alanine Aminotransferase Increased	0	-	1	1.7% (1/58)

Table S3. Continued from previous page.

System Organ Class Preferred Term	Antibiotic Period 1+2 Events	 %(n/N)	IVIg Period 1+2 Events	 %(n/N)
Aspartate Aminotransferase Increased	0	-	1	1.7% (1/58)
Biopsy Prostate	1	1.8% (1/56)	0	-
Blood Creatinine Increased	2	3.6% (2/56)	0	-
Laboratory Test Abnormal	0	-	1	1.7% (1/58)
Metabolism And Nutrition Disorders	2	3.6% (2/56)	0	-
Hypercholesterolaemia	1	1.8% (1/56)	0	-
Hyperkalaemia	1	1.8% (1/56)	0	-
Musculoskeletal And Connective Tissue Disorders	9	12.5% (7/56)	17	22.4% (13/58)
Arthralgia	2	1.8% (1/56)	3	3.4% (2/58)
Arthritis	0	-	1	1.7% (1/58)
Arthropathy	0	-	1	1.7% (1/58)
Back Pain	2	3.6% (2/56)	2	3.4% (2/58)
Bursitis	0	-	1	1.7% (1/58)
Intervertebral Disc Protrusion	0	-	1	1.7% (1/58)
Muscle Spasms	1	1.8% (1/56)	0	-
Musculoskeletal Pain	1	1.8% (1/56)	0	-
Musculoskeletal Stiffness	0	-	1	1.7% (1/58)
Myalgia	1	1.8% (1/56)	3	5.2% (3/58)
Osteitis	0	-	1	1.7% (1/58)
Osteoporosis	1	1.8% (1/56)	0	-
Pain In Extremity	0	-	1	1.7% (1/58)
Pain In Jaw	0	-	1	1.7% (1/58)
Systemic Lupus Erythematosus	1	1.8% (1/56)	0	-
Tendonitis	0	-	1	1.7% (1/58)
Neoplasms Benign, Malignant And Unspecified (Incl Cysts And Polyps)	2	3.6% (2/56)	1	1.7% (1/58)
Basal Cell Carcinoma	1	1.8% (1/56)	0	-
Gastrointestinal Tract Adenoma	0	-	1	1.7% (1/58)
Lung Neoplasm	1	1.8% (1/56)	0	-
Nervous System Disorders	11	14.3% (8/56)	71	48.3% (28/58)
Aphasia	0	-	1	1.7% (1/58)
Disturbance In Attention	0	-	1	1.7% (1/58)
Dizziness	0	-	1	1.7% (1/58)
Essential Tremor	0	-	1	1.7% (1/58)
Headache	8	12.5% (7/56)	60	39.7% (23/58)

Table S3. Continued from previous page.

System Organ Class Preferred Term	Antibiotic Period 1+2		IVIg Period 1+2	
	Events	%(n/N)	Events	%(n/N)
Migraine	0	-	2	1.7% (1/58)
Sciatica	1	1.8% (1/56)	0	-
Sensory Disturbance	0	-	1	1.7% (1/58)
Sinus Headache	2	1.8% (1/56)	3	3.4% (2/58)
Syncope	0	-	1	1.7% (1/58)
Psychiatric Disorders	1	1.8% (1/56)	2	3.4% (2/58)
Attention Deficit/Hyperactivity Disorder	1	1.8% (1/56)	1	1.7% (1/58)
Depression	0	-	1	1.7% (1/58)
Renal And Urinary Disorders	0	-	2	3.4% (2/58)
Nephrolithiasis	0	-	2	3.4% (2/58)
Reproductive System And Breast Disorders	1	1.8% (1/56)	0	-
Fibrocystic Breast Disease	1	1.8% (1/56)	0	-
Respiratory, Thoracic And Mediastinal Disorders	4	7.1% (4/56)	17	19.0% (11/58)
Asthma	0	-	2	3.4% (2/58)
Chronic Obstructive Pulmonary Disease	1	1.8% (1/56)	5	3.4% (2/58)
Cough	0	-	1	1.7% (1/58)
Diaphragmatic Hernia	1	1.8% (1/56)	0	-
Dyspnoea	1	1.8% (1/56)	5	6.9% (4/58)
Nasal Discomfort	0	-	1	1.7% (1/58)
Nasal Obstruction	1	1.8% (1/56)	1	1.7% (1/58)
Rhinitis Allergic	0	-	1	1.7% (1/58)
Rhinorrhoea	0	-	1	1.7% (1/58)
Skin And Subcutaneous Tissue Disorders	3	3.6% (2/56)	26	24.1% (14/58)
Angioedema	1	1.8% (1/56)	0	-
Blister	0	-	2	1.7% (1/58)
Dyshidrosis	0	-	1	1.7% (1/58)
Eczema	0	-	1	1.7% (1/58)
Exfoliative Rash	0	-	1	1.7% (1/58)
Hyperhidrosis	0	-	1	1.7% (1/58)
Ingrowing Nail	0	-	1	1.7% (1/58)
Night Sweats	0	-	1	1.7% (1/58)
Pruritus	0	-	2	3.4% (2/58)
Psoriasis	1	1.8% (1/56)	0	-
Rash	0	-	12	10.3% (6/58)
Rash Pruritic	0	-	2	1.7% (1/58)

Table S3. Continued from previous page.

System Organ Class Preferred Term	Antibiotic Period 1+2		IVIg Period 1+2	
	Events	**%(n/N)**	**Events**	**%(n/N)**
Skin Depigmentation	0	-	1	1.7% (1/58)
Skin Lesion	0	-	1	1.7% (1/58)
Urticaria	1	1.8% (1/56)	0	-
Surgical And Medical Procedures	2	3.6% (2/56)	1	1.7% (1/58)
Central Venous Catheterisation	0	-	1	1.7% (1/58)
Intra-Uterine Contraceptive Device Insertion	1	1.8% (1/56)	0	-
Mammoplasty	1	1.8% (1/56)	0	-
Vascular Disorders	1	1.8% (1/56)	5	6.9% (4/58)
Hot Flush	0	-	1	1.7% (1/58)
Hypertension	1	1.8% (1/56)	2	1.7% (1/58)
Phlebitis	0	-	2	3.4% (2/58)

Source: Table 14.3.01.04 MedDRA version 14.1 was used

CHAPTER 8

Risk factors associated with a poor clinical outcome of interstitial lung disease in common variable immunodeficiency.

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ABSTRACT

Background: Untreated granulomatous and lymphocytic interstitial lung disease (gl-ILD) can become a major cause of morbidity and mortality among patients with common variable immunodeficiency. However, predictive risk factors for progressive disease are lacking.

Objectives: This study aimed to identify demographic, clinical, radiographic and immunologic markers typical for gl-ILD and gl-ILD progression.

Methods: Patients who were treatment naive and who had radiological and pulmonological data recorded before treatment commenced were included in this study (n = 92). A poor clinical outcome was defined as 1) oxygen dependency, 2) right heart failure or pulmonary hypertension, or 3) FVC or $DLCO_{SB}$ <60%. Logistic linear regression was used for risk factor assessment.

Results: Occurrence of gl-ILD was associated with an increased female:male ratio, splenomegaly, lymphadenopathy, auto-immune cytopenias, increased CD4+CD45RO+ T cells, reduced switched-memory B cells and increased $CD21^{low}$ B cells. Index pulmonary function tests were impaired in 86% of patients and deteriorated in 53% of patients. Moreover, 32% of patients had an impaired resting O2 saturation or capillary partial oxygen pressure during the study. CT scans showed a relapsing remitting pattern. Impaired $DLCO_{SB}$ correlated with more gl-ILD related CT lesions and impaired FVC correlated with more bronchiectasis. Hepatopathy, increased ILD scores on CT and higher circulating NK cell numbers predicted a poor clinical outcome.

Conclusions gl-ILD is characterized by specific demographic, clinical, radiographic and immunologic markers and progresses in the majority of patients. Higher initial ILD scores on CT, higher NK cell counts and hepatopathy can potentially be used to identify patients at risk for a poor clinical outcome and could guide future screening efforts and decisions towards early treatment initiation.

Keywords: CVID; Granulomatous and lymphocytic interstitial lung disease; Hartmann score; corticosteroids; gl-ILD; immune dysregulation; observational trial; pulmonary function tests; quality of life.

Abbreviations

CVID common variable immunodeficiency, CT computed tomography, DLCO diffusion lung capacity for CO, FVC functional vital capacity, gl-ILD granulomatous lymphocytic interstitial lung disease, LIP lymphocytic interstitial pneumonitis, pO2 partial pressure of oxygen, SpO2 pulse oxygen saturation, TLC total lung capacity

INTRODUCTION

Interstitial lung disease (ILD) is seen in about 10-25% of patients with common variable immunodeficiency (CVID) and often presents with complex pulmonary interstitial pathologies.[1–5] ILD in CVID is a diffuse lung parenchymal disease and is distinct from airway disease.[6–8] Airway disease consists of bronchial wall thickening and bronchiectasis and often coincides with ILD in CVID.[7,9–14] The term granulomatous lymphocytic interstitial lung disease (gl-ILD) has been coined first in 2004 by Bates et al comprising granulomatous disease, lymphocytic interstitial pneumonia (LIP), follicular bronchiolitis (FB), and organizing pneumonia and in a subgroup of patients also fibrotic changes, although the term is still controversial.[1,15] gl-ILD is regarded as the pulmonary manifestation of a general lymphoproliferative and/or granulomatous course of the underlying immunodeficiency.[16]

There is absent consensus on the need for histological confirmation of gl-ILD.[17–19] Because of the invasive nature of the biopsy procedure, several efforts have been made to describe gl-ILD specific radiological patterns that characterize the severity of gl-ILD such as nodules, ground glass changes and reticulation in more detail.[6,8,20–25]

Additionally, some studies have investigated the use of biomarkers to monitor disease progression in gl-ILD.[17] Multiple studies have shown that sIL-2R and IgM potentially correlate with disease activity.[26–31] Moreover, one study has shown that gl-ILD patients have elevated levels of markers that indicate T cell activation, pulmonary epithelium injury and extra cellular matrix remodeling compared to patients with CVID suffering from other non-infectious complications.[31] Others studies have shown that serum B cell activating factor (BAFF) and neopterin levels correlate with gl-ILD activity.[27,28]

Finally, multiple studies have investigated a combination of biomarkers and clinical symptoms that might be indicative of gl-ILD.[17] Multiple studies have shown that an elevated percentage of $CD21^{low}$ B cells and the presence of auto-immune cytopenias and/or splenomegaly are predictive for gl-ILD in CVID patients.[5,32–34] Moreover, a reduced forced vital capacity (FVC) and a reduced diffusion capacity of the lung for carbon monoxide (DLCO) have both been indicated as potential diagnostic markers in separate studies.[5,34] Other potential criteria included the presence of polyarthritis or lymphadenopathy, a reduced CD8+ count, low IgA levels and an increased Baumann's GLILD score.[5,32–34]

Initial reports described a poor prognosis for CVID patients with gl-ILD with a median survival of 13.7 years compared to other CVID patients (28.8 years).[1] The clinical course of gl-ILD has been further described in a small case series which had reported CT progression of fibrosis over 3 years for 8/14 patients without reporting the course of pulmonal functional testing adequately.[21] Another study reported an average annual decline in FEV1 of 36 ml and in FVC of 39 ml for CVID patients.[35] Moreover, a more recent study retrospectively analyzed the natural course of gl-ILD, describing 15 patients that were naïve to immunosuppression and found progressive loss of lung function in 9 of these patients without giving details on the associated changes in CT.[26] Two other cohorts reported on the prevalence of progressive gl-ILD, defined as progressive loss of lung function and/or progression on CT and found progressive gl-ILD in 52% and 59% of patients [27,30]

Thus, without treatment gl-ILD deteriorates in 44% of treatment naive patients and progressive gl-ILD has been described in 56% of all gl-ILD patients. However, studies that investigate associated clinical risk factors that can determine in which patients gl-ILD will deteriorate, using a multivariable approach are lacking. That is why we were interested to determine the natural course of gl-ILD in a larger cohort of patients and identify factors associated with gl-ILD progression or poor outcome using a multivariable approach.

We therefore designed the study of interstitial lung disease in patients with antibody deficiency (STILPAD), in order to shed more light on this potentially life-threatening manifestation of CVID. Here we report the natural course of the disease in 93 patients of 14 European centers and report risk factors associated with deterioration of gl-ILD.

METHODS

Type of study

Patients included in this study were derived from the Study of Interstitial Lung Disease in Primary Antibody Deficiency (STILPAD), which was a non-interventional, multicenter European study (DRKS-ID: DRKS00000799). It consisted of a retrospective and a prospective analysis of up to five years for which 145 participants were recruited. Retrospective documentation started at the first event of either gl-ILD diagnosis, documented pulmonary function test (PFT), or chest computed tomography (CT). The prospective observation period started June 29, 2012 and was performed over a period of up to 5 years (last patient out January 19, 2018).

Study population

Patients written consent was obtained, patients without legal capacity who were unable to understand the nature, significance and consequences of the study were excluded. Inclusion criteria were:

- Diagnosis of CVID according to the ESID criteria from 1999[36] (IgG ≤ 2 SD below the mean for age and a marked decrease in at least one of the isotypes IgM or IgA; onset of immunodeficiency at greater than 2 years of age; absent isohemagglutinins and/or poor response to vaccines; exclusion of defined causes of hypogammaglobulinemia)
- Chest CT positive for nodules, lines or ground-glass signs compatible with interstitial lung disease or granuloma
- Male or female aged ≥ 18 years
- No active immunosuppressive treatment at the start of the study
- At least one FVC or $DLCO_{SB}$ measured during follow-up or available baseline and follow-up CT.

Exclusion criteria were:

- Therapy with rituximab or chemotherapy before to the start of observation.
- Therapy with other immunosuppressants 5 years prior to the start of observation.

Observation ended when a patient was started on immunosuppressants or when the end of the prospective observation phase was reached.

Pulmonary function evaluation

The FVC and $DLCO_{SB}$ and the pO2 and oxygen saturation were used in this study. If available the index PFT and blood oxygen values (within 1 year of gl-ILD diagnosis) were used, otherwise the first documented variable (baseline) was used. Additionally, the final documented variable (follow-up) before the end of the study was used for evaluation of outcome.

PFT and blood oxygen values were graded following general practice:[37]

- $DLCO_{SB}$ % of expected: 0: normal >=75%, 1: mild (60-75%), 2: clinically relevant (<60%) impairment
- FVC % of expected: 0: normal >=70%, 1: mild (60-70%), 2: clinically relevant (<60%) impairment

- Abnormal blood oxygen values defined as arterial or capillary pO2 <70 mmHg or – in case of lacking values oxygen saturation <94%.

Relevant decline of PFT was defined according to previously published criteria as FVC ≥10% decline and $DLCO_{SB}$ ≥15% decline, repectively.[28,38]

Radiographic evaluation

If available the index CT (within 1 year of gl-ILD diagnosis) was used, otherwise the first documented CT scan (baseline) was used. Additionally, the final documented CT scan (follow-up) was used for evaluation of outcome. CT scans were centrally analyzed by two blinded experts according to the Hartmann Score, as previously described.[23,25] Progression on CT was defined according to previously published criteria (≥5 point increase on follow-up scan).[28]

Secondary parameters

Secondary parameters consisted of clinical parameters, immunoglobulins, T and B cell subsets and sIL-2R levels, For a complete list of included parameters see supplementary table 1.

Statistics

Statistical analysis was performed using R Studio (version 2023.03.0). Categorical data are summarized by the number and percentage of subjects in each category. Continuous variables are summarized by descriptive statistics, including median and minimum the range or interquartile range (IQR). Fisher's exact test was used for comparison of categorical variables. Proportions found in this study were compared to published data from other retrospective and prospective cohorts using Chi-squared tests. The Wilcoxon rank-sum test was used for comparison of continuous variables. Spearman's rank correlation was used to generate a correlation matrix and principal component analysis (PCA) was performed. Logistic linear regression was used for multivariable risk factor assessment. For univariable risk factor assessment, $p<0.2$ was deemed relevant. For the remaining analysis $p<0.05$ was deemed significant.

RESULTS

Epidemiological, clinical and immunological description of cohort

A treatment-naive phase was described in 119 of the 146 patients included in STILPAD, after evaluation of inclusion and exclusion criteria 93 of 119 patients

were eligible for this study. The cohort comprised 92 Caucasian patients and one Turkish patient. Patients had been recruited in four German centers (n = 41), in seven UK centers (n = 35) and in three French centers (n = 17), compiling a total of 511 treatment-naive patient years. Median age at time of inclusion was 48 years (19 – 78). Median age at CVID diagnosis was 39 years (9 – 67) and median age at gl-ILD diagnosis was 43 years (13 – 74). During the entire study period, 20 patients (22%) remained treatment-naive with a median follow-up of 7.6 years (range 3.6- 22.4 years), 73 were started on immunosuppressive therapy during the observation period after a median follow-up time of 3 years (range 0 -21.5 years). For 44 patients (47%), clinicians reported that treatment was initiated specifically for gl-ILD. Corticosteroids were given most frequently as initiation therapy and were given to 61 patients (84%), followed by rituximab (5 patients, 7%) and disease modifying antirheumatic drugs (4 patients, 5%). Genetics were performed in 54 patients and revealed a genetic diagnosis in 8 patients (5 CTLA4 and 3 NFKB1) and 2 patients that were carrier of the TACI susceptibility gene.

When epidemiological and clinical parameters were compared between the different countries we found that patients from the UK had a shorter median follow-up time (2.6 years), which was significantly shorter than French patients (7.42 years) and possibly shorter than German patients (4.26 years, Table 1). Moreover, patients from the UK received general immunosuppressive treatment more frequently, but treatment was not specifically initiated for gl-ILD more frequently. Moreover, an underlying genetic diagnosis was more frequent among UK patients. Splenomegaly, lymphadenopathy and hepatopathy were reported less frequently among patients from the UK. Additionally, German patients reported a history of chronic obstructive pulmonary disease more frequently.

We compared this cohort of CVID patients with gl-ILD to general CVID cohorts and found that more women than men were affected, there was a higher prevalence of splenomegaly, lymphadenopathy and autoimmune cytopenia, but not enteropathy or hepatopathy.[39,40] We compared clinical parameters of our cohort to compiled clinical parameters of previously reported adult gl-ILD CVID cohorts.[1,5,27,30,32–34,41–46] We found that gl-ILD diagnosis preceded CVID diagnosis in fewer patients and lymphadenopathy and potentially lymphoma was less frequently reported in our cohort (Table 2).

Table 1: Demographic data of patients included in this cohort across the three participating countries.
If data was missing the n is reported in brackets.

	Entire Cohort (n)	Germany (n)	England (n)	France (n)	p-value
n	93	41	35	17	
Median age at start of study	48	43	52	52	**0.03**
Median age at gl-ILD diagnosis (years)	43	39	45	45	0.12
Sex (% F)	63	61	66	64	0.9
Median follow-up (years)	4.04	4.26	2.57	7.42	**<0.01**
IST started	78	73	91	65	**0.05**
gl-ILD therapy started	47	51	49	35	**0.53**
CVID associated genetic diagnosis (%)	19 (54)	10 (30)	39 (18)	0 (6)	**0.04**
Asthma (%)	10	5	9	24	0.08
COPD (%)	5	12	0	0	**0.04**
Former smoker (%)	39	34	40	59	0.18
Splenomegaly (%)	83	93	66	94	**<0.01**
Lymphadenopathy (%)	61	76	34	82	**<0.01**
Auto-immune cytopenia (%)	44	51	46	24	0.15
Hepatopathy (%)	17	21	9	41	**0.02**
Enteropathy (%)	16	20	14	12	0.81
Lymphoma (%)	4	0	9	6	0.14

Table 2: Demographic and clinical and immunological data of gl-ILD patients included in this cohort, compared to historical gl-ILD data.
CVID = common variable immunodeficiency, F = female, GD = granulomatous disease, gl-ILD = granulomatous lymphocytic interstitial lung disease,

Parameter	In this cohort	In historical cohorts (n)	Included patients	Included studies	p-value
n	93	372		[1,5,26,27,30,32–34,41–46]	
Median age of gl-ILD onset (years)	43	29 – 56	gl-ILD	[1,5,27,30,32–34,42,43,45,46]	NA
gl-ILD >1 year diagnosed before CVID (%)	11	45 (78)	gl-ILD/ GD	[3,44]	**<0.01**
Sex (% F)	63	59 (357)	gl-ILD	[1,5,27,30,32–34,41–46]	0.48
Splenomegaly (%)	83	78 (343)	gl-ILD	[1,5,26,30,32–34,41–46]	0.21
Lymphadenopathy (%)	61	74 (217)	gl-ILD	[26,30,33,34,41,43–45]	**0.02**
Auto-immune cytopenia (%)	44	50 (294)	gl-ILD	[5,27,30,32–34,41–44]	0.31
Hepatopathy (%)	17	22 (219)	gl-ILD	[5,26,30,32,33,42,43,45,46]	0.33
Enteropathy (%)	16	21 (203)	gl-ILD	[4,5,30,32,33,42]	0.30
Lymphoma (%)	4	10 (218)	gl-ILD	[1,26,30,32,34,42,45]	0.07

In regard to the immune phenotype, immunoglobulins measured at CVID diagnosis were available for 83 patients. When compared to the general CVID cohort, gI-ILD patients more frequently had IgA levels below the limit of detection.[47] Additionally, 78 patients were stratified according to EuroClass and we found that gI-ILD patients had reduced frequencies of switched memory B cells and increased frequencies $CD21^{low}$ B cells compared to the general CVID cohort (Table 3).[39] T cell phenotyping data was available for 58 patients and reduced naive CD4 (<10%) was found more frequently in gI-ILD patients, compared to CVID patients.[47] Signs of a combined immunodeficiency (CD4+ T-cell counts <200/ and CD4 naive <10%) were found in only 2 patients.

Table 3: Immunological data of gI-ILD patients included in this cohort, compared to historical CVID data. *If data was missing the n is reported in brackets.*

Parameter	In this cohort	In historical cohorts (n)	Included studies	p-value
Immunoglobulins at CVID diagnosis	83	268	[39]	
IgG <1.0 g/L	36	34 (188)	[39]	0.63
IgA <0.2 g/L	77	50 (257)	[39]	**<0.01**
IgM <0.07	40 (81)	30	[39]	0.09
EuroClass stratification (N)	78	303	[39]	
Switched memory B cells <2% (%)	76	58	[39]	**<0.01**
$CD21^{low}$ cells >10% (%)	71	57 (229)	[39]	**0.03**
Normal B phenotype (%)	8	26 (229)	[39]	**<0.01**
T cell phenotyping (N)	58	238	[47]	
CD4+ <200 (%)	4	3	[47]	0.71
CD4+ naive <10% (%)	45	25	[47]	**0.01**

Histology was available for 27 patients and confirmed the diagnosis in 14 patients (52%). GLILD-score items were available for 72 patients and the GLILD score was ≥50 in 50 patients (69%). Eleven patients with histologically proven gI-ILD had an assessable GLILD score and the score was ≥50% in nine patients (82%).

Pulmonary function and radiological manifestations at the time of diagnosis of gI-ILD

The index FVC was available for 63 patients and the index $DCLO_{SB}$ was available for 52 patients, combined data was available for 51 patients. Median index FVC was 92% of expected (IQR: 69.5 – 108.5) and median index $DLCO_{SB}$ was 71% of expected (IQR: 59 – 82). In 19 patients (29%) both parameters were normal, in 10 patients (22%) only the FVC was impaired, in 23 (35%) only the $DLCO_{SB}$ and in 9 (14%) both parameters were impaired. Index measurements of pO2 or oxygen saturation at rest without application of oxygen were available for 24 patients of whom only

three showed impairment. The FVC was impaired in 2 hypoxemic patients and the $DLCO_{SB}$ was impaired in 1 hypoxemic patient.

Index CT scans were available for 40 patients. After scoring, nodules were found to be the most common feature of index CTs and were found in 38 patients (95%) with a median score of 8 (IQR 4 - 12) in regard to number and 7 (IQR 4 – 11.75) in regard to size. Nodules are evenly distributed throughout all lobes with no clear preferred lobes for large nodules. Ground glass changes were detected in 30 patients (75%) with a median score of 2.5 (IQR 0.75 - 5) affecting the lower lobes more often. Mostly mild reticulation with distortion was seen in the baseline CT of 33 patients (83%) with a median score of 1 (IQR 0 - 3) and a predominance for the middle and lower lobes. In 78% nodules and ground glass changes co-occur, nodules without any further interstitial pattern are seen in 8%.

Focusing on more advanced disease with scores of ≥2, prevalence of nodules amounts to 95% and of ground glass to 58%, an overlap of nodules with interstitial changes is seen in 86%. Ground glass changes or reticulation scores ≥ 2 occur without nodules in only 2 patients. Ground glass sum scores ≥6 only occur in patients with high scores in the number of nodules (5 and above) and the size of nodules (6 and above).

Mild coexisting bronchiectasis was seen on baseline CT in 83% of patients with a median score of 2 (IQR 1 - 4) for large and 1 (IQR 0 – 3) for small bronchiectasis and ≥2 was present in 66% of gl-ILD patients.

Description of the natural course

We examined first the course of the pulmonary parameters by comparing first and last measurement during the untreated phase of disease when at least > 6 months apart. FVC data was available for 62 patients and $DLCO_{SB}$ data was available for 56 patients. Median time between tests was 4.1 years (IQR 2.0 – 6.1). The FVC (-2%, p=0.04) and $DLCO_{SB}$ (-4%, p<0.01) worsened over time (Figure 1A-B). FVC showed a relevant decline in 16 (26%) and $DLCO_{SB}$ in 17 patients (27%), 6 patients showed a combined decline of FVC and $DLCO_{SB}$. Overall, 68 patients had a follow-up FVC or $DLCO_{SB}$ measurement available and 72% (49) of these measurements were impaired. Of these patients, 46 had an impaired $DLCO_{SB}$ (<75% of expected), 14 had an impaired FVC (<70% of expected); 11 of these patients had a combined impairment. Approximately half of patients (48%) that showed a decline in PFT were subsequently put on specific treatment for gl-ILD, however nearly the same

proportion of patients that showed no decline in PFT ended the observation period because of treatment for gl-ILD (43%).

Second, we examined the course of resting pulse oxygen saturation (spO_2) and partial pressure of oxygen (pO_2). The spO_2 data was available for 44 patients and the pO_2 data was available for 26 patients. Median time between tests was 3.1 years (IQR 2.0 – 5.8). We found a significant decline of both the spO_2 (-1%, $p<0.03$) and the pO_2 (-7 mmHg, $p<0.01$, Figure 1C-D) The spO_2 declined in 20 patients (45%) and the pO_2 declined in 22 patients (85%). 53 patients had their spO_2 or pO_2 measured before the end of the study. Of these patients, 7 had impaired saturation (<94%) and 10 had impaired blood oxygen levels (<70 mmHg); 4 of these patients had a combined impairment. Approximately half of hypoxemic patients (47%) were started on treatment for gl-ILD, but hypoxemic patients were not treated more often than normoxic patients (47% vs 47%).

Third, we examined spO_2 and pO_2 during exercise, for which baseline and follow-up data had been collected for 19 patients. We did not find a significant decline of spO_2 or pO_2 under exercise during follow-up. However, at least one spO_2 was measured in 32 patients and in 14 patients these were <94%. Moreover, in 12 patients the resting spO_2 was normal, while the spO_2 during exercise was impaired. A similar pattern was seen for pO_2, which was measured at least once in 21 patients and impaired in 10 patients. The resting pO_2 of 4 patients was normal, while the pO_2 during exercise was impaired. Thus, it seems that spO_2 and pO_2 are more frequently impaired during exercise than during resting. Half of hypoxemic patients during exercise (50%) specifically treated for gl-ILD, but hypoxemic patients were not treated more often than normoxic patients (50% vs 20%).

Finally, we examined the course of CT findings, which were available for 39 patients. Median time between tests was 4.1 years (IQR 1.9 – 5.5). We did not find significant changes of the ILD sub scores or the total ILD score (Figure 2E-H) as the observed changes varied strongly between patients. Ground glass opacities improved in 16 patients, remained stable in 7 patients and worsened in 16 patients. Nodules improved in 20 patients, remained stable in 2 patients and worsened in 17 patients. Reticulation improved in 18 patients, remained stable in 7 patients and worsened in 14 patients. Overall, 8 patients had a high ILD score of ≥18 on their first CT, but not on their follow-up CT, while 4 patients had an ILD score of <18 on their first CT, but of ≥18 on their follow-up CT. Overall, an ILD score ≥18 was found on the follow-up CT in 46% patients compared to 63% of patients at baseline.

Figure 1: Pulmonary function tests and O_2 levels deteriorate significantly over time. *Forced vital capacity (FVC, A), diffusion capacity for carbon monoxide in a single breath ($DLCO_{SB}$, B), oxygen saturation (C), partial O_2 pressure (D), ground glass sum score (E), nodules sum score (F), reticulation sum score (G) and total ILD score (H) were all compared between baseline (T1) and follow-up (T2). FVC, $DLCO_{SB}$, oxygen saturation and partial O_2 pressure all significantly declined (paired Wilcoxon-signed rank test).*

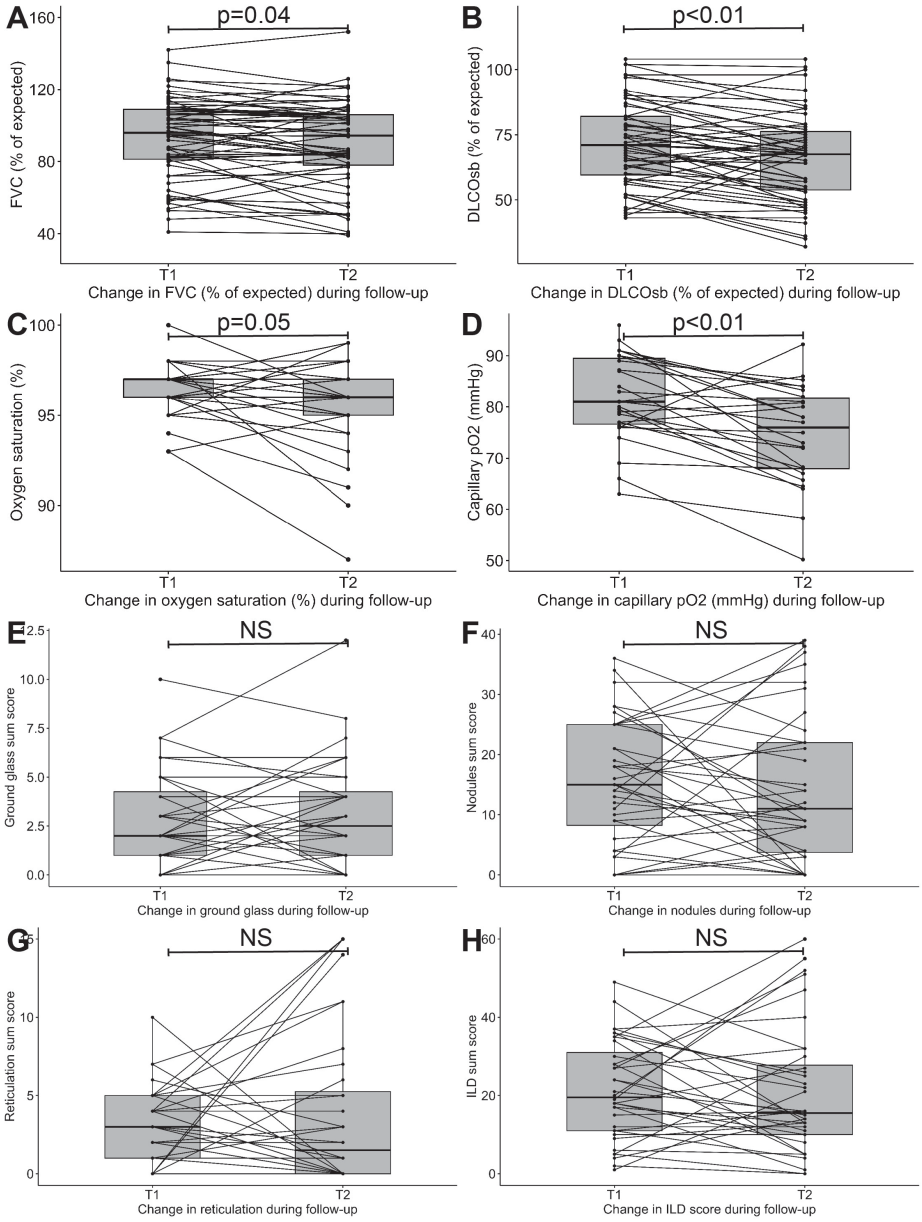
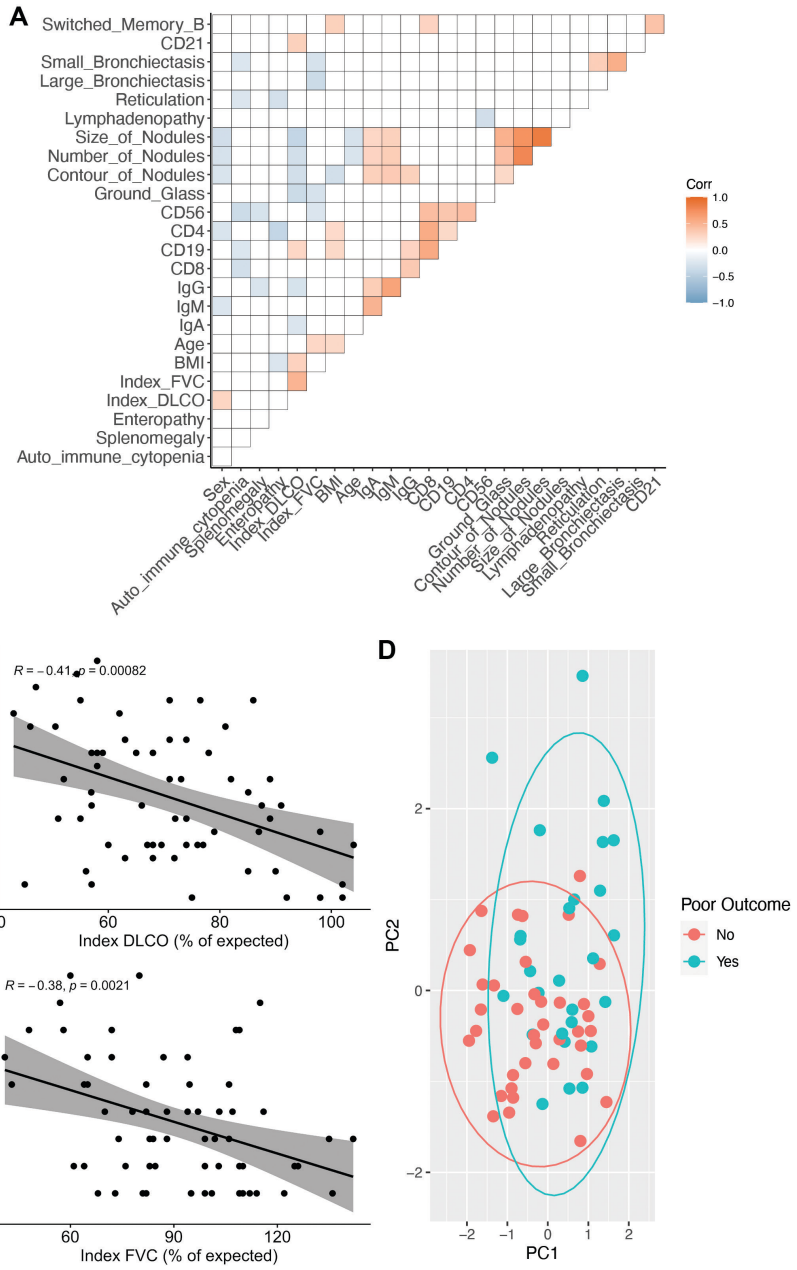


Figure 2: Diffusion capacity of the lung (DLCO) and forced vital capacity (FVC) correlate with ILD and bronchiectasis related CT items. *(A) A correlation matrix shows that impaired $DLCO_{SB}$ correlates with increased nodules and ground glass on CT and impaired FVC correlates with more bronchiectasis on CT. (B-C) Examples DLCO and FVC correlations with their respective CT items are shown. (D) DLCO clustered together with ILD related CT items (PC1) and FVC with bronchiectasis related CT items and could moderately identify patients with a poor clinical outcome.*



Correlation of pulmonary and non-pulmonary parameters

We analyzed the correlation of 25 pulmonary and non-pulmonary parameters (Figure 2A-C) in 64 patients for whom a baseline CT was available. Unfortunately, sIL-2R and O_2 measurements could not be included in the correlation matrix because of missing data. Here we found that female patients had more nodular lung disease on the index CT and a worse $DLCO_{SB}$. Moreover, a worse $DLCO_{SB}$ correlated with a worse FVC, increased $CD21^{low}$ cells, decreased BMI, increased nodular lung disease and increased ground glass on the index CT. A worse FVC correlated with increased NK cells, increased ground glass and more bronchiectasis on the index CT. Additionally, higher immunoglobulins at CVID diagnosis correlated with more nodular disease on the index CT.

As sIL-2R was measured in only 31 patients, we analyzed correlations between sIL-2R and pulmonary parameters separately. Here we found that sIL-2R levels correlated with nodular disease on follow-up CT (R=0.51, p=0.03) and potentially with nodular disease on baseline CT (R=0.44, p=0.06).

Factors associated with a poor clinical outcome

Next, we further analyzed factors associated with a poor clinical outcome. A poor clinical outcome was defined as: 1) need of oxygen therapy, 2) diagnosis of right heart failure or pulmonary hypertension, or 3) baseline or follow-up values of FVC or $DLCO_{SB}$ <60% of expected. A poor clinical outcome was assessable in 90 patients and found in 42 patients (47%). A poor outcome was found in 26 patients during baseline assessment and in an additional 16 patients during follow-up assessment. Patients with a poor outcome were treated with immunosuppressive therapy just as often as patients without a poor outcome (79% vs 77%).

First, we investigated the relationship between pulmonary and non-pulmonary variables and poor clinical outcome, we performed a principal component analysis (PCA) with oblique rotation on the same dataset. However, there were too few samples to explain all 25 variables, expressed by the Kaiser-Meyer-Olkin (KMO) measure of common variance. The variables with the poorest common variance were excluded, which were the presence of enteropathy, lymphadenopathy, auto-immune cytopenias, splenomegaly and reduced switched memory B cells, the reticulation score and age. This resulted in acceptable PCA prerequisites with a KMO measure of 0.63 and a highly significant Bartlett's Test. Six PCs were able to explain most of the variance within the dataset (70%). We found that the baseline $DLCO_{SB}$ clustered together with sex, ground glass and the nodular lung disease

scores, while baseline FVC clustered together with the bronchiectasis scores. Together these PCs were moderately capable of identifying patients with a poor clinical outcomes (Figure 2D).

Second, clinical, radiographic and laboratory parameters were compared in a univariable analysis (Table 4). Here we found demographic, clinical, radiographic and immunological factors that were potentially associated with a worse outcome.

Third multivariable analysis was performed and we found that the presence of hepatopathy and increased ILD sum scores at baseline were significant predictors of a worse outcome and higher NK cell counts were a potential predictor. Unfortunately, the T and B cell subsets could not be included for multivariable analysis, because of missing data and several parameters had to be excluded from the final model because of a high variance influencing factor.

Table 4: Potential predictors of poor clinical outcome (FVC<60%, $DLCO_{SB}$<60%, PHT or O_2 dependent).

	Good outcome	Poor outcome	p-value
n	**48**	**42**	
Significant Parameters			
Sex (% F)	52	76	0.02
Enteropathy or hepatopathy (%)	21	43	0.02
Hepatopathy (%)	13	45	0.03
Median ground glass sum score	1	3	<0.01
Median nodules sum score	14	25	0.02
Median total ILD score	19	30	<0.01
Median NK cells (per µL)	70	109	0.04
Median IgG (g/L)	0.1	0.2	0.02
Potential Parameters			
Auto-immune cytopenia (%)	50	36	0.17
Median reticulation with distortion	2	1	0.19
Median CD4+ cells (per µL)	572	744	0.17
Median naive CD4%	9.2	20.2	0.14
Median memory CD4%	87.9	75.6	0.20
Median naive CD8%	19	9.8	0.12
Reduced class-switched memory B cells (%)	82	68	0.07
Increased $CD21^{lo}$ B cells (%)	66	75	0.13
Median class-switched memory B cells (%)	0.7	1	0.11
Median IgM+ memory B cells (%)	9	6	0.2
Median IgM (g/L)	0.1	0.2	0.07

Finally, we calculated cut-offs for the ILD score and the NK cell counts. Since the AUC of both the ILD score (0.64) and NK cell counts (0.59) was relatively poor, we decided to build two prognostic models, one that favored specificity and on that favored sensitivity (Table 5). We found that the presence of either hepatopathy or a combination of an ILD score > 20 and NK cell counts >195 was 88% specific (CI; 76 – 96) and 38% sensitive (CI; 24 – 54) for a poor outcome. We also found that either the presence of hepatopathy or an ILD score >26 or NK cell counts >200 was 53% specific (CI; 38 – 67) and 88% sensitive (CI; 74 – 96) for a poor outcome. We also performed an identical analysis for potential risk factors for progression on PFT or CT, but we did not find significant predictors.

Table 5: Prognostic model for poor gl-ILD outcome, patients at risk had at least 1 positive criterion.
ILD = interstitial lung disease, NK = natural killer, SEN = sensitivity, SP = specificity

	Criterion 1	Criterion 2	Criterion 3	SEN	SP
Model 1	Hepatopathy	ILD score >20 and NK>195	-	38%	88%
Model 2	Hepatopathy	ILD score >26	NK >200	88%	53%

DISCUSSION

Here we describe the largest cohort of treatment naive gl-ILD patients to date, explore the natural course of gl-ILD and show factors associated with a poor clinical outcome. Additionally, we show that the manifestation of gl-ILD is associated with specific demographic, clinical, radiographic and immunological factors and a correlation of CT findings and PFT.

Index PFT were impaired in the majority of patient (78%) and the $DLCO_{SB}$ was affected most frequently (61%). This was also described in a previous study, where $DLCO_{SB}$ was found to be an important predictor of gl-ILD.[34] Moreover, the $DLCO_{SB}$ was already significantly affected (<60% of expected) at index in 44% of patients. Additionally, we found that nodules were the most common feature on index CT and were seen in 95% of patient and high ILD scores (≥18) at index were also seen in the majority of patients (68%).

We find PFT and/or CT progression in the majority (68%) of patients. This finding has previously also been described by three smaller cohorts.[21,27,30] In some patients (32%), gl-ILD even leads to impaired resting pulse oxygen saturation or partial pressure of oxygen. While PFT's often either worsened or remained stable, CT

scans showed the relapsing/remitting pattern that has previously been described for gl-ILD.[28] Moreover, we identified that 46% of patients had poor PFT results, were oxygen dependent or had pulmonary hypertension, which was defined as a poor clinical outcome in this study. Additionally, 18% of patients progressed and fulfilled these criteria during follow-up.

Additionally, we find that high ILD scores at baseline combined with increased NK cells and the presence of hepatopathy can be predictive of a poor clinical outcome. However, it is unclear whether these markers also play a role in gl-ILD pathophysiology. A high ILD score on CT is probably a marker of high disease activity, and might reflect nodular infiltration that causes pulmonary damage that leads to a poor clinical outcome.[23] This is also reflected by the direct correlation between $DLCO_{SB}$ and gl-ILD CT parameters, shown in this study. On the other hand, NK cells have not yet been described to play a role in the pathogenesis of gl-ILD and have not been found in pulmonary biopsies of gl-ILD patients. [17] However, NK cells have been implicated in other auto-immune diseases and increased and decreased NK cell counts and altered NK cell function has been described to play a role in these diseases.[48] Finally, advanced hepatopathy has been previously linked to impaired pulmonary function caused by hepatopulmonary syndrome and might therefore further aggravate the clinical outcome of gl-ILD.[49] Alternatively, it could also be an intermediate factor that reflects the severity of immune dysregulation in CVID patients. At different cut-offs, high ILD scores, increased NK cells and hepatopathy can potentially be used in the future to guide screening efforts and decision towards early initiation of gl-ILD specific treatment, which potentially could prevent complications in gl-ILD patients.[6]

Like our meta-analysis of previous cohorts, [1,5,27,30,32–34,41–46] we find an increased female:male ratio for gl-ILD compared to the general CVID population.[3,34,40] We confirm the previously reported association between other non-infectious complications and gl-ILD, especially splenomegaly, lymphadenopathy and auto-immune cytopenias. [1,5,27,30,32–34,41–46] In line with the data of the Italian cohort, we show a similar age of CVID onset in gl-ILD patients and an association of gl-ILD with decreased switched memory B cells, increased $CD21^{low}$ B cells and decreased $DLCO_{SB}$.[34] gl-ILD is also associated with a decrease of circulating CD4+CD45RA+ naive T cells.[42,50] gl-ILD diagnosis was made before, at the same time or after CVID diagnosis, but unlike previous reports preceded CVID diagnosis in only 11% of our patients.[3,44] Despite differences in the onset and severity of gl-ILD we find that gl-ILD patients show similar gl-ILD associated factors that consist of radiographic

evidence of ILD, impaired $DLCO_{SB}$, splenomegaly, lymphadenopathy and auto-immune cytopenias and B and T cell aberrancies.

However, some limitations of this study should be taken into consideration. Firstly, the mixed design of a retrospective and prospective phase led to missing data. We resolved this by using linear stochastic regression to impute the missing data. This method might have resulted in an overidentification of interrelationships, since this approach reduces the statistical noise in the dataset. Moreover, for some variables there was too much missing data for linear stochastic regression to be applied, leading to exclusion of potential risk factors from the multivariable analysis. Second, the small sample size and the use of non-parametric tests might have resulted in type 2 errors. Third, besides sIL-2R our study did not account for other known biomarkers for gl-ILD and gl-ILD progression like IgM, BAFF and neopterin. The risk factors identified in this study need replication in an independent cohort, preferably in a prospective cohort where CT and PFT outcomes and biomarker levels are measured at regular intervals

Despite these limitations, we conclude that gl-ILD is a clinical manifestation of a systemic immune dysregulation in CVID characterized by gl-ILD associated CT findings, impaired $DLCO_{SB}$, the presence of other non-infectious complications and T and B cell dysregulation, which progresses in the majority of patients. Patients at risk for a poor outcome can potentially be identified by higher initial ILD scores on CT, higher NK cell counts and hepatopathy. These clinical markers could potentially guide future screening for high-risk gl-ILD patients and also decisions towards early initiation of treatment.

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SUPPLEMENTARY TABLES

Supplementary Table 1

Parameter	Retrospective (if available)	Baseline visit	Regular visit (every 6-12 months)	Additional visit[a]	Final visit (5 years after baseline)
Demographic characterstics		x			
Genetics					x (if available)
Medical history	x	x	x	x	x
infections	x (only major events)	x	x	x	x
Need of LTOT		x	x	x	x
Smoking status	x	x	x	x	x
Immunosupressive therapy	x	x	x	x	x
IgGRT	x	x	x	x	x
Concomitant medication		x	x	x	x
Physical examination		x	x	x	x
Ig levels	x (initial values)	x	x	x	x
immunophenotyping	x (latest values, prior to relevant IS)				
Biomarkers sIL2R, neopterin, IL6	x (sIL2R)	x	x	x	x
PFT (DLCOcSB, DLCOc/VA, TLC, FVC, FEV1, pO2 or O2 saturation at rest)	x (all available measurements)	x	x (every 12 mos.)	x	x
Exercise testing	x (all available measurements)	x	x (every 24 mos.)	x	
Chest CT scan (digital version)	all available scans, indication at discretion of investigator (recommended every 24 mos.)				
BAL analysis	x (all available measurements)				
lung histology	Centrally performed re-analysis of all available samples				
QoL CRQ-SAS, SGRQ		x	x	x	x
QoL SF-36		x	x (every 12 mos.)		x

[a] visits being scheduled because of pulmonary complaints, change of immunosuppression or 3-6 months after increase of immunosuppression

LTOT long term oxygen therapy, *IgGRT* IgG replacement therapy, *Ig* immunoglobulin, *sIL2R* soluble interleukin-2 receptor, *IL6* interleukin-6, *PFT* pulomonary function testing, *DLCOcSB* diffusion capacity of the lung single breath, *DLCOc/VA* diffusion capacity of the lung per alveolar volume, *TLC* total lung capacity, *FVC* functional lung capacity, *FEV1* forced expiratory volume in one second, *BAL* bronchoalveolar lavage, *QoL* quality of life, *CRQ-SAS* Chronic Respiratory Questionnaire Self-Administered Standardized, *SGRQ* St George's Respiratory Questionnaire , SF-36 36-Item Short Form Health Survey

CHAPTER 9

Treatment Strategies for GLILD in Common Variable Immunodeficiency: A Systematic Review

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ABSTRACT

Introduction: Besides recurrent infections, a proportion of patients with Common Variable Immunodeficiency Disorders (CVID) may suffer from immune dysregulation such as granulomatous-lymphocytic interstitial lung disease (GLILD). The optimal treatment of this complication is currently unknown. Experienced-based expert opinions have been produced, but a systematic review of published treatment studies is lacking.

Goals: To summarize and synthesize the published literature on the efficacy of treatments for GLILD in CVID.

Methods: We performed a systematic review using the PRISMA guidelines. Papers describing treatment and outcomes in CVID patients with radiographic and/or histologic evidence of GLILD were included. Treatment regimens and outcomes of treatment were summarized.

Results: 6124 papers were identified and 42, reporting information about 233 patients in total, were included for review. These papers described case series or small, uncontrolled studies of monotherapy with glucocorticoids or other immunosuppressants, rituximab monotherapy or rituximab plus azathioprine, abatacept, or hematopoietic stem cell transplantation (HSCT). Treatment response rates varied widely. Cross-study comparisons were complicated because different treatment regimens, follow-up periods, and outcome measures were used. There was a trend towards more frequent GLILD relapses in patients treated with corticosteroid monotherapy when compared to rituximab-containing treatment regimens based on qualitative endpoints. HSCT is a promising alternative to pharmacological treatment of GLILD, because it has the potential to not only contain symptoms, but also to resolve the underlying pathology. However, mortality, especially among immunocompromised patients, is high.

Conclusions: We could not draw definitive conclusions regarding optimal pharmacological treatment for GLILD in CVID from the current literature since quantitative, well-controlled evidence was lacking. While HSCT might be considered a treatment option for GLILD in CVID, the risks related to the procedure are high. Our findings highlight the need for further research with uniform, objective and quantifiable endpoints. This should include international registries with standardized data collection including regular pulmonary function tests

(with carbon monoxide-diffusion), uniform high-resolution chest CT radiographic scoring, and uniform treatment regimens, to facilitate comparison of treatment outcomes and ultimately randomized clinical trials.

Keywords: systematic review, immunodeficiency, common variable immunodeficiency, CVID, granulomatous lymphocytic interstitial lung disease, GLILD, treatment

INTRODUCTION

Common variable immunodeficiency disorders (CVID) are the most common symptomatic primary immunodeficiencies, with an estimated incidence between 1:10.000 and 1:50.000.[1] Patients typically suffer from recurrent respiratory tract infections, such as bronchitis, sinusitis, otitis media and pneumonia. Moreover, they are often affected by immune dysregulation, a term which encompasses auto-immune manifestations, auto-inflammatory disease and lymphoproliferation, and by malignancy.[2] Infection risk in CVID can be minimized by means of antimicrobial prophylaxis and immunoglobulin replacement therapy (IgRT). In contrast, immune dysregulation is much more difficult to prevent and treat, and remains a major cause of morbidity and mortality.[3-6]

Granulomatous lymphocytic interstitial lung disease (GLILD) is one of the complications of CVID and is considered the pulmonary manifestation of multi-system immune dysregulation. GLILD occurs in approximately 10-20% of patients with CVID and was reported to be responsible for a reduction in life expectancy of more than 50% after diagnosis in adult patients, from a median of 28.9 to 13.7 years.[6,7] GLILD may be asymptomatic, or may present with non-specific symptoms such as cough and dyspnea on exertion.[4] Small or large nodules, consolidations and ground glass abnormalities in the lower regions of the lung on high-resolution CT-scan are highly suggestive of GLILD.[8] The diagnosis can be confirmed by biopsy (via video-assisted thoracoscopic surgery, transbronchial or percutaneous intervention) and FDG-PET-CT may be used for the identification of active inflammatory lesions elsewhere.[4,9] The combination of routine chest CT-scans and pulmonary function tests, including specifically diffusing capacity of carbon monoxide, should be used to identify GLILD in CVID and monitor disease progression.[9]

The etiology of GLILD is still poorly understood. Maglione and colleagues (2019) pointed out that patients with X-linked agammaglobulinemia (XLA) have severe antibody deficiency that is even more pronounced than CVID but only rarely develop GLILD. Patients with XLA lack mature B-cells, whereas patients with CVID have peripheral B-cells, although often with impaired function, suggesting that B-lymphocytes may play a causative role in GLILD development. Indeed, lymphocytic (but not the granulomatous) progression has been associated with an increased production of B-cell activating factor (BAFF), which in turn leads to activation of the anti-apoptotic factor Bcl-2, thereby promoting B-cell survival

as well as an increase of IgM producing CD21 low B-cells.[10] Unger et al. (2018) linked the expansion of CD21low B-cells with disproportionally high numbers of Th1 cells and increased interferon-γ production, probably reflecting the aberrant combined T-B interaction in the pathogenesis of interstitial lung disease in CVID.[11] It has also been suggested that viral infections may trigger GLILD, as Wheat et al. (2005) identified a correlation between human herpesvirus 8 (HHV8) infection and the disease.[12] However, since the publication of the original article describing this correlation, no further evidence has been provided for this hypothesis. Finally, an association between interstitial lung disease and an increased relative abundance of Streptococcus in the oropharyngeal microbiome in CVID was recently identified.[13]

The treatment of GLILD mostly consists of immunosuppressive medication, in addition to IgRT and other supportive measures such as physiotherapy. According to the British Lung Foundation/United Kingdom Primary Immunodeficiency Network Consensus Statement (2017), glucocorticoids are the first line of therapy for GLILD. Most clinicians agree that azathioprine, mycophenolate mofetil (MMF) and rituximab are second-line choices when glucocorticoids are not effective or when attempting to spare their use. Although alternative medication may also be prescribed, there is no consensus about the use of other biologic therapies or disease-modifying anti-rheumatic drugs (DMARDs).[9]

Current GLILD treatment guidelines are based on expert opinion rather than on robust scientific evidence. An objective review of the existing evidence is needed to minimize potential biases associated with expert opinion, and to identify knowledge gaps. Therefore, our aim was to systematically review the existing literature on treatment of GLILD in CVID patients. To the best of our knowledge, this is the first systematic review on that topic.

METHODS

We searched PubMed and EMBASE for publications on treatment of GLILD in CVID patients (last search on March 27th 2020, see appendix for search string). Articles describing patients with CVID and GLILD who were treated with pharmacological therapy and/or a hematopoietic stem cell transplantation (HSCT) were included. Improvement of disease activity parameters (symptoms, pulmonary function tests and radiological findings) and mortality served as outcomes.

We focused our search on patients with CVID and GLILD. Studies describing patients with monogenetic diseases causing a CVID-like phenotype (such as CTLA-4 haploinsufficiency and LRBA deficiency) were included.

The consensus GLILD definition of the British Lung Foundation/United Kingdom Primary Immunodeficiency Network was used: "GLILD is a distinct clinic-radio-pathological interstitial lung disease occurring in patients with CVID, associated with a lymphocytic infiltrate and/or granuloma in the lung, and in whom other conditions have been considered and where possible excluded".[9] Only articles that reported radiological findings on a CT-scan or histological analysis of biopsies compliant with this definition of GLILD were included.

All non-English articles were excluded for purposes of practicality. Conference abstracts, while read and taken into consideration, were excluded from the review as they were not peer-reviewed.

Two independent investigators (O.L. and B.S.) selected articles on the basis of title and abstract. Blinding of the investigators was achieved by inserting all articles in a common online database (Rayyan), which has a blinding feature and allows each researcher to select articles independently of the other. Ultimately, the selection of articles of each researcher was compared to the other. If there were any selection discrepancies, the articles were discussed until a unanimous decision about in- or exclusion could be made. Data were extracted from the eligible full-text articles using a standardized data extraction sheet. The extracted data were summarized descriptively and reported in tables. We could not conduct meta-analyses because the selected articles contained insufficient quantitative data.

If the use of multiple treatment regimens in one patient was reported, the effect of the treatment regimens was evaluated separately. When escalation or switching of treatment was deemed necessary by the authors, the previous regimen was deemed insufficient. To evaluate the effect of treatment regimens, both qualitative and quantitative assessments of GLILD activity were analyzed. Descriptive improvement of pulmonary function tests, radiological findings and symptoms (e.g. "shortness of breath", "coughing") were used for the qualitative evaluation of disease activity. Significant improvement was defined as a relapse-free improvement of at least one of these parameters and no deterioration of the other parameters. Pre- and post-treatment pulmonary function test results were used for the quantitative evaluation of disease activity, and significant improvement of

pulmonary function was here defined as a 10% increase in at least one pulmonary function test parameter.

Overall risk of bias of each study was assessed by means of a self-designed tool based on the PRISMA guidelines.[14] This tool took into account the quality of the studies (based on the number of patients and controls, and on descriptions of outcomes, medication dosages and follow-up procedures) and possible confounders (smoking, age, comorbidity, and results of genetic testing). Each study was assigned a rating for each of these categories, 'good' (+) if the highest quality standard was attained with clear quantitative outcomes, 'intermediate' (+/-) if some information was reported but quantitative measures were lacking, and 'insufficient' (-) if the information was not reported at all. The overall risk of bias was determined as follows: 'high risk of bias' if the study had four or more insufficient or eight or more intermediate judgements; 'intermediate risk of bias' if the study was marked insufficient on two to four items or intermediate on four to eight items; and 'low risk of bias' if the study had only one insufficient judgement or a maximum of three intermediate judgements.

The level of evidence for each study and the degree of recommendation in clinical practice were determined following the criteria formulated by the Centre for Evidence Based Medicine.[15]

RESULTS

The search identified 6124 articles on PubMed and EMBASE and seven additional papers via snowballing (Figure 1). After removal of duplicates, 5304 articles were screened, 65 full-text papers were read, and 42 articles were deemed eligible. 233 patients were described in total. The findings are summarized below, sorted by treatment modality. Qualitative and quantitative lung function findings are shown in Figure 2.

There were three papers describing GLILD in patients with B lymphocyte related primary antibody deficiency other than CVID (such as IgA or IgG subclass deficiency, or selective antibody deficiency for polysaccharide antigens). These articles are listed in the supplementary material (Table S1).

Figure 1: PRISMA flow chart for article inclusion.

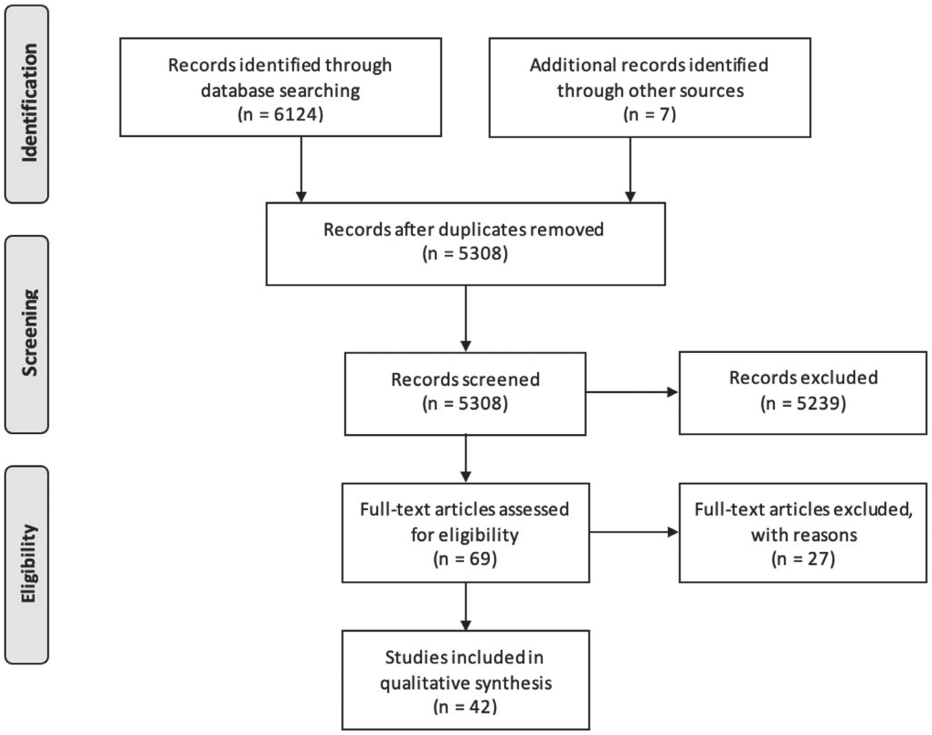
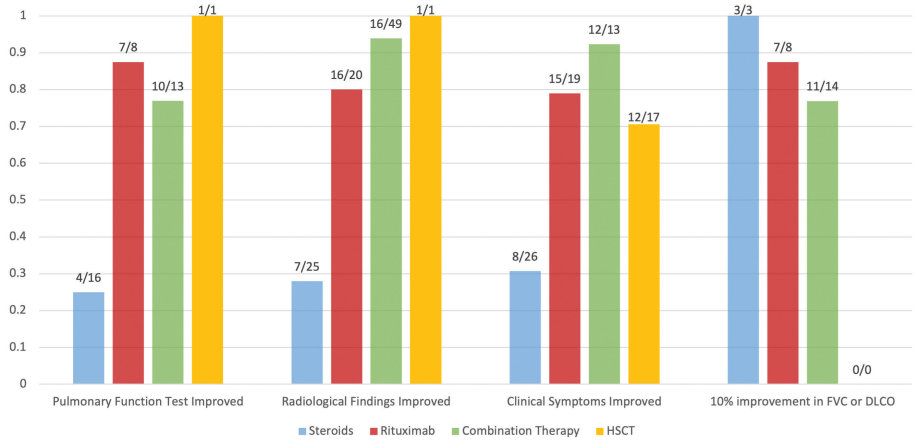


Figure 2: Comparison of the available qualitative and quantitative outcomes of studies that reported on patients (N) treated with steroids, rituximab monotherapy and rituximab combination therapy. *The proportion of patients that had a qualitatively reported improvement of pulmonary function tests, radiological findings and the proportion that had a quantitative improvement of their forced vital capacity (FVC) or diffusion capacity of the lung for carbon monoxide (DLCO) of 10% after therapy is shown. Due to a lack of quantitative data, statistics could not be performed.*



Glucocorticoids

Glucocorticoids have been identified as the first line treatment for GLILD by the British Lung Foundation/United Kingdom Primary Immunodeficiency Network (2017).[9]

Six articles specifically reported on the use of glucocorticoids for the treatment of GLILD in patients with CVID, as shown in Table 1. The first report dates back to 1982 and describes the case of a woman who was treated with high-dose prednisone for six weeks. Symptoms initially subsided but relapsed when the medication was tapered.[16] Ten additional studies included glucocorticoid treatment as one of several therapies (Table 2 & Table 3). Five of these reported no effect of glucocorticoids,[17-22] one reported relapse after initial remission[23] and four reported treatment success.[24-27] The article by Kanathur et al. (2000) is particularly interesting as it describes a case in which glucocorticoids initially failed to have any effect at all but were associated with the resolution of symptoms when paired with splenectomy.[28]

Table 1: Studies reporting treatment of GLILD in PID with corticosteroids.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Boujaoude et al., 2013	Case study	32-year-old woman with CVID and GLILD	Prednisone at a dose of 60 mg daily, duration not mentioned	None	Improvement of CS, PFT and RF	FVC: 0.61 L increase ((% predicted increased by 19%), FEV1: 0.48 L increase
Guerrini et al., 2018	Case study	20-year-old woman with CVID and GLILD	Corticosteroids, exact duration not mentioned	None	Improvement of CS and RF	Not mentioned
Kohler et al., 1982	Case study	35-year-old woman with CVID and GLILD	Prednisone at a dose of 60 mg daily for six weeks, after which tapering was initiated	None	Improvement of PFT and RF, relapse when tapering was attempted	FVC: 0.98 L increase (% predicted increased by 28%), FEV1: 0.7 L increase

Table 1: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Kanathur et al., 2000	Case study	67-year-old man with CVID and GLILD	Splenectomy and prednisone at a dose of 60 mg daily for 18 months	None	No effect of prednisone at first, after splenectomy prednisone was continued, resulting in improvement of CS and RF	Not mentioned
Kaufman et al., 1991	Case study	26-year-old woman with CVID and GLILD	Prednisone at a dose of 60 mg daily for a few months, exact duration not mentioned	None	Improvement of PFT and RF	FVC: 0.08 L increase (% predicted increased by 2%) FEV1: 0.01 L increase (no change in % predicted)), DLCO: 2.9 ml/mm/mmHg (% of predicted increased by 13%)
Wislez et al., 2000	Case study	68-year old woman with CVID and GLILD	Prednisone at a dose of 0.75 mg per kg daily, then tapering to 5 mg daily over the course of two months and stopping completely eight months later.	None	Improvement of CS and RF, but relapse upon interruption of glucocorticoids. Improvement of symptoms upon reintroduction of glucocorticoids.	Not mentioned

Abbreviations: CVID: common variable immunodeficiency; CS: clinical symptoms; DLCO: diffusing capacity; FVC: forced vital capacity, FEV1: forced expiratory volume in 1 second; GLILD: granulomatous-lymphocytic interstitial disease; MMF: mycophenolate mofetil; MTX: methotrexate; PFT: pulmonary function tests; RAG: recombination-activating gene; RF: radiological findings.

Conventional Disease Modifying Anti Rheumatic Drugs (DMARDs)

Besides glucocorticoids, other immunosuppressants for the treatment of GLILD have been evaluated (Table 2). Examples encountered in the literature included methotrexate (MTX), cyclophosphamide, mycophenolate (MMF), azathioprine, cyclosporin, hydroxychloroquine, tacrolimus and sirolimus.

Boursiquot et al. (2013) assessed the efficacy of both MTX and cyclophosphamide in the treatment of GLILD. The researchers prospectively followed 59 patients with CVID, of whom 30 had GLILD. Different treatment regimens were initiated in 25 patients with CVID and GLILD (Table 2). Complete remission was obtained in three (out of 13) patients who were treated with glucocorticoids, one (out of one) who was treated with MTX and one (out of five) who was treated with cyclophosphamide. Ten patients had a partial response and the remainder showed no effect at all.[29]

Other articles reported the use of MMF for the treatment of GLILD. Bucciol et al. (2015) described three patients with GLILD. Glucocorticoids were ineffective, but a switch to MMF resulted in stabilization of symptoms and improvement of clinical and radiologic findings in all three cases.[30] More evidence was provided by Tashtoush et al. (2016), who published a case report about a 51-year old woman with CVID and GLILD. This patient achieved remission after induction therapy with glucocorticoids for 3 months and MMF maintenance therapy for 9 months.[31]

As emerged from the Delphi Study of the British Lung Foundation/United Kingdom Primary Immunodeficiency Network, azathioprine is another drug that is often used for the treatment of GLILD. An article dating back to 1996 by Sacco et al. reported the case of a six-year-old girl with CVID and severe GLILD. The patient was treated with glucocorticoids with good effect, but tapering of the medication resulted in disease relapse. This prompted the physicians to add azathioprine, which halted disease progression. The combination of prednisone and azathioprine was maintained for three years, after which they were tapered to 5 mg every other day and 0.75 mg per kg daily, respectively.[23]

Albeit less frequently reported, several articles describe the use of cyclosporine for the treatment of GLILD. Davies et al. (2000) reported the case of a 34-year old woman with CVID and GLILD who responded well to glucocorticoid therapy, but had recurrent relapses after tapering. The patient was eventually treated with cyclosporine, with good effect.[19] Similar results were observed by Cha et al. (2006):a patient with CVID and concomitant GLILD was initially treated with glucocorticoids, but achieved disease remission only when therapy was switched to cyclosporin.[17]

Deya-Martinez et al. (2018) showed that the immunosuppressant sirolimus can be useful in the treatment of GLILD. A boy with CVID and GLILD, who had been

previously treated with rituximab and who had relapsed, was switched to sirolimus monotherapy and achieved remission of symptoms.[32]

Two articles reported the use of DMARDs for the treatment of GLILD in relatively large patient series. Both papers described variable regimens of multiple drugs, without mentioning the outcomes.

Ardeniz (2009) described the long-term follow up of a group of 37 patients with CVID and granulomatous disease, of which 20 patients had GLILD. Patients were treated with a different combination of drugs, including glucocorticoids, cyclosporine, hydroxychloroquine, infliximab, etanercept and rituximab. Outcomes were not clearly reported. Over the follow-up period of 25 years, 10 of the 37 patients included in the study died. Of those, at least five had GLILD.[33]

Bouvry (2013) compared outcomes of CVID patients with GLILD with those of patients with sarcoidosis. Patients were treated with different immunosuppressants over the course of the study. Results were not clearly reported, the main difference between the two groups was that patients with CVID and GLILD had worse outcomes than those with sarcoidosis.[34]

Table 2: Studies reporting treatment of GLILD in antibody deficiencies with various immunosuppressants.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Ardenitz et al., 2009	Prospective follow up cohort study	37 patients with CVID and granulomatous disease, of which 20 also had GLILD	Splenectomy was performed in nine patients, 29 patients were given glucocorticoids, with or without other therapies, 10 subjects were also given one or more additional immune suppressants: hydroxychloroquine (five subjects), cyclosporine (three subjects), azathioprine (two subjects), methotrexate (two subjects), infliximab (one subject), and etanercept (one subject). One patients was administered rituximab. Five patients received no treatment. Duration of treatments varied. Treatment of 13 patients with GLILD was specifically reported. Patient 04: prednisone and hydroxychloroquine Patient 08: cyclosporine at a dose pf 100 mg twice daily, years of prednisone, IV glucocorticoids Patients 11: monthly oral and IV glucocorticoids Patient 14: chronic prednisone at a dose of 20 mg daily Patient 20: oral prednisone for 12 months Patients 21 oral prednisone for 12 months Patient 24: infliximab, hydroxychloroquine at a dose of 200 mg twice daily for 15 years Patient 28: MTX at a dose of 7.5 mg weekly for 12 months, hydroxychloroquine at a dose of 200 mg twice daily for five years Patient 34: years of prednisone, hydroxycholoroquine Patient 35: years of steroids at a dose of 10 mg every two days Patient 36: oral steroids at a dose of 5 mg daily for one week, COX2 inhibitors	Patients with same disease received different treatments	Outcomes were not reported for single patients. 10 (28.5%) patients died (seven of pulmonary complications and at least five with GLILD), rituximab led to resolution of autoimmunity, unclear how other drugs were effective	Not mentioned

Table 2: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Boursiquot et al., 2013	Prospective follow up cohort study	59 patients with CVID of which 30 also had GLILD	25 treatment regimens were noted. Oral corticosteroids were administered to 13 patients for a median of 18 months, six received cyclophosphamide for a median of six months, hydroxychloroquine was used in four cases for a median of 13.5 months, rituximab in three for a median of six months. MTX for a median of 38 months, thalidomide for a median of two months, infliximab and azathioprine were each used in two patients for a median of 31 and 18 months respectively. Cyclosporine, Interferon alpha, MMF and sirolimus were used in one patient each, for a median of 12, six, 20 and 12 months	31 patients with CVID who did not receive any treatment	Complete remission was obtained in three patients who were treated with corticosteroids, one who was treated with MTX and one who was treated with cyclophosphamide. 10 patients had a partial response and 10 had no effect at all	Not mentioned
Bouvry et al., 2013	Prospective follow up cohort study	20 patients with CVID and GLILD	17 patients received IVIg, 15 corticosteroids, three others not specified immunosuppressants and two hydroxychloroquine, duration not specified	60 patients with sarcoidosis	Six of the patients with CVID and GLILD died, all of the patients with sarcoidosis were still alive	Not mentioned
Bucciol et al., 2015	Case study	Three patients with CVID and GLILD: 23-year-old man, 18-year-old man and 4-year-old girl	Corticosteroids, duration not specified MMF, duration not specified	None	Resistance to steroids or relapse despite steroids. Stabilization of CS and improvement of RF after MMF administration	Pt 1; FVC: (% predicted decreased by 7%, FEV1: (% predicted decreased by 4%. Pt 2: Pre-treatment data not mentioned, FVC after treatment 60% of predicted FEV1 after treatment 68% of predicted Pt 3: not mentioned

Table 2: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Cha et al., 2006	Prospective follow-up cohort study	15 patients with various underlaying diseases (one had CVID)and GLILD)	Corticosteroids, MTX, colchicine, azathioprine, cyclophosphamide and cyclosporin. Patient with GLILD: corticosteroids and MTX, later switched to cyclosporin, duration not mentioned	None	Patient with CVID: still alive, no effect of corticosteroids and MTX, improvement of CS and PFT when switched to cyclosporin	Not mentioned
Davies et al., 2000	Case study	34-year-old woman CVID and GLILD	Prednisone at a dose of 40 mg daily Cyclosporin at a dose of 125 mg daily	None	No effect of prednisone, improvement of CS and RF on cyclosporin A	FVC: 0.71 L increase ((% predicted increased by 30%), FEV1: 0.6 L increase
Deya-Martinez	Case study	2 patients (12-year-old boy with CVID and GLILD and 16-year-old girl with Kabuki syndrome and GLILD)	Pt 1: rituximab at a dose of 375 mg per m2 weekly for 4 weeks twice. MMF and sirolimus at dose of 2.5 mg/m2 daily, duration not specified Pt 2: sirolimus, duration not specified	None	Pt 1: Good effect of rituximab initially, but relapse six months after treatment. Improvement of with MMF and sirolimus. Pt 2: Improvement of RF with sirolimus	Not mentioned.
Franxman et al., 2014	Case series	3 patients with CVID and GLILD (14-year-old female, 55-year-old female and a 16-year-old male)	Pt 1: Corticosteroids and MMF, dose and duration not specified. Infliximab 5 mg/kg every 4 weeks for 4 months Pt 2: Corticosteroids and plaquenil, dose and duration not specified. Infliximab 5 mg/kg every 4 weeks for 6 months Pt 3: Corticosteroids, dose and duration not specified. Infliximab 5 mg/kg every 4 weeks for 5 months		Pt 1: No effect of corticosteroids, after initiation of infliximab steroids could be tapered and there was improvement of CS, PFT and RF. Pt 2: Decline of RF PFT and CS during corticosteroid therapy. Improvement of CS & PFT. Discontinuation of treatment due to possibly treatment related skin lesions. Pt 3: Relapse upon tapering of steroids. Improvement of CS & PFT and successful taper of steroids after infliximab introduction	Pt 1; FVC: increased by 22%, FEV1: increased by 20% Pt 2; FVC: increased by 6%, DLCO: increased by 33%. Pt 3; FVC: increased by 35%

Table 2: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Sacco et al., 1996	Case study	Six-year-old girl with CVID and GLILD	Corticosteroids at a dose of 2 mg per kg daily for two weeks, after which tapering was started. A dose of 0.75 mg per kg daily was maintained for three years, until it was further tapered to 0.17 mg per kg per day. Azathioprine at a dose of 1.5 mg daily, for the duration of three years, after which the dose was tapered to 0.75 mg per kg per day	None	Improvement of clinical symptoms and RF with corticosteroids only, but relapse when tapering. Addition of azathioprine stabilised situation	Not mentioned
Tashtoush et al., 2016	Case study	51-year-old patient with CVID and GLILD	Prednisone at a dose of 0.5 mg per kg daily for 3 months MMF at a dose of 1000 mg daily for nine months	None	Improvement of CS and RF after 3 months	Not mentioned
Thatayatikom et al., 2005	Case study	22-year-old man with CVID and GLILD	High-dose methylprednisolone Infliximab at a dose of 10 mg daily for six weeks. After relapse treatment with infliximab was re-initiated at a dose of 5 mg daily for nine months	None	No effect of methylprednisolone, improvement after addition of infliximab, then relapse with interruption of treatment. Again, improvement of CS and RF after therapy re-initiation	Not mentioned

Abbreviations: CVID: common variable immunodeficiency; CS: clinical symptoms; DLCO: diffusing capacity; FVC: forced vital capacity, FEV1: forced expiratory volume in 1 second; GLILD: granulomatous-lymphocytic interstitial disease; MMF: mycophenolate mofetil; MTX: methotrexate; PFT: pulmonary function tests; RAG: recombination-activating gene; RF: radiological findings.

Biologicals

Biologicals, also known as biological medicinal products, are drugs which are (partially) produced by living organisms by means of recombinant DNA technologies.[35] For GLILD specifically, infliximab, rituximab and abatacept have been used.

Infliximab

Infliximab is a monoclonal antibody that binds to TNFα and blocks signaling, thus interfering with a central mechanism of inflammation.[36] Thatayatikom et al. (2005) reported a 22-year-old man with CVID and life-threatening GLILD, who was first unsuccessfully treated with glucocorticoids, but achieved remission after treatment with infliximab for nine months.[20] Additionally, Franxman, Howe & Baker (2014) described three patients who all showed remission of GLILD on CT scan and pulmonary function tests, after 4 months, 8 months and 5 months of treatment, respectively.[37]

Rituximab

Rituximab is a monoclonal antibody that depletes B-cells, by binding to CD20 molecules on their surface.[38] Seven studies focused on rituximab monotherapy for GLILD (Table 3). Arraya (2018), Cereser (2019), Ng (2019) and Tessarin (2019) all reported cases of patients with CVID and GLILD who were successfully treated with rituximab monotherapy (at a dose of 375 mg/m2 weekly for four weeks).[39-42] Maglione et al. (2019) followed 73 patients for 18 months: 44 patients had CVID only, 14 had concomitant stable GLILD, and 15 had concomitant progressive GLILD. 11 of the 15 patients with progressive GLILD were treated with rituximab at a dose of 375 mg/m2 weekly for four weeks: all experienced stabilization or improvement of disease activity, however four relapsed 18 months after completion of therapy.[10]

Of particular interest is the study by Zdziarsky and Gamian's (2019), describing a 25-year old woman with CVID and GLILD who was treated with rituximab monotherapy at a relatively low dose of 150 mg/m2 weekly for six weeks because of risk of infection.[22] This resulted in incomplete remission of clinical symptoms, and the patient relapsed six months later. Treatment with rituximab was repeated, this time at a dose of 375 mg/m2, resulting in complete remission for a period of 30 months.

Table 3: Studies reporting treatment of GLILD in PID with rituximab.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Arraya et al., 2019	Case report	57-year-old female with CVID and GLILD	Rituximab at a dose of 375 mg/m2 weekly for four cycles. Three cycles were used for induction, a yearly cycle was used for maintenance for 8 years.	None	Improvement of RF	Not mentioned
Ceserer et al., 2019	Case series	Three patients with CVID and GLILD (38- and 56-year-old women, 44-year-old man)	Rituximab at a dose of 375 mg/m2 weekly for four cycles. At total of 16 infusions was given	None	Improvement of CS, PFT and RF	Pt 1; FVC: 0.37 L increase ((% predicted increased by 11%), DLCO: 0.6 ml/mm/mmHg increase ((% predicted increased by 8%), FEV1: 3.04 L increase ((% predicted increased by 38%) Pt 2; FVC: 0.36 L increase ((% predicted increased by 24%), DLCO: 0.4 ml/mm/mmHg increase ((% predicted increased by 7%), FEV1: 0.19 L increase ((% predicted increased by 12%) Pt 3: FVC: 0.25 L decrease ((% predicted decreased by 4%), DLCO: 0.9 ml/mm/mmHg increase ((% predicted increased by 9%), FEV1: 0.36 L decrease ((% predicted decreased by 7%).
Maglione et al., 2019	Prospective cohort study	11 patients with CVID and progressive GLILD	Rituximab at a dose of 375 mg/m2 weekly for four cycles	44 patients with CVID but no GLILD, 14 patients with CVID and stable GLILD and four patients with CVID and progressive GLILD	Improvement of CS and RF. Relapse of 4 patients.	Not mentioned

Table 3: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Ng et al., 2019	Case study	Two patients with CVID and GLILD (36-year-old man and 33-year-old woman)	Corticosteroids, duration not specified Rituximab at a dose of 375 mg/m2 weekly for four cycles with a four- to six-month interval. A total of 16 infusions was given	None	Corticosteroids led to short-lived improvement of CS, rituximab led to improvement of CS and RF	Not mentioned
Tessarin et al., 2019	Case study	37-year-old woman with CVID and GLILD	Rituximab at a dose of 375 mg/m2 every four weeks, weekly for four cycles with a four to six month interval	None	Improvement of CS and RF	Not mentioned
Vitale et al., 2015	Case study	37-year-old woman with CVID and GLILD	High-dose corticosteroids, duration not specified Rituximab at a dose of 375 mg/m2 every four weeks, weekly for four cycles with a four to six month interval	None	Corticosteroids had no direct effect, addition of rituximab led to improvement of CS, PFT and RF	Not mentioned
Zdziarsky and Gamian, 2019	Case study	25-year-old woman with CVID and GLILD	Methylprednisone at a dose of up to 50 mg daily, duration not specified Rituximab at a dose of 150 mg/m2 weekly for six cycles and later at a dose of 375 mg/m2 every 21 days for four cycles with a six-month remission interval	None	No effect of corticosteroids, improvement after first underdosed cycle of rituximab followed by relapse, improvement of CS and RF after second cycle of rituximab	FVC: 1.21 L increase

Abbreviations: CVID: common variable immunodeficiency; CS: clinical symptoms; DLCO: diffusing capacity; FVC: forced vital capacity, FEV1: forced expiratory volume in 1 second; GLILD: granulomatous-lymphocytic interstitial disease; PFT: pulmonary function tests; RF: radiological findings

Combination chemotherapy with rituximab and azathioprine

Eight studies evaluated combination chemotherapy with rituximab and azathioprine (Table 4). The rationale behind this combination chemotherapy is that B- and T-lymphocytes are targeted simultaneously.[18] Chase and colleagues (2013) were the first ones to pioneer this approach. They performed a longitudinal prospective cohort study in which they followed seven patients with CVID and GLILD, who were treated with intravenous rituximab and oral azathioprine for 18 months. All patients experienced some degree of improvement in radiological findings.[18] These results were confirmed by Pathria (2016), Routes (2017), Limsuwat (2018) and Tillman (2019), who reported successful treatment of patients with CVID and GLILD with combination chemotherapy.[43-46] Vitale et al. (2015), reported successful addition of combination therapy with rituximab to glucocorticoid treatment in a 17-year old patient with CVID and GLILD after initial unresponsiveness to glucocorticoid monotherapy.[21] Jolles' (2015) and Sood's (2018) articles showed that azathioprine can be replaced by other drugs with similar mechanisms of action. For example, Jolles et al. (2015) described a 51-year old woman with CVID and GLILD treated with a combination of rituximab and MMF, because of intolerance of azathioprine. Five months into treatment, the patient experienced an improvement of symptoms, alongside better pulmonary function and radiologic results.[47] Sood et al. (2018) reported an improvement of GLILD related symptoms in the case of a 16-year old boy with 22q.11 deletion syndrome who was treated with rituximab and 6-mercaptopurine.[48] One additional article by Verbsky et al. was added to the review despite its publishing date (June 2020) being after the last literature search (March 2020). We choose to mention this article, because the planned publication of the paper was known to the authors at the time of the literature search and, most importantly, because its results are highly relevant for this systematic review. The authors performed a retrospective chart review of 39 patients with CVID and GLILD who were treated with a combination of rituximab and azathioprine or rituximab and MMF. The median follow-up period was four years. 37 patients were included in the final analysis and of those 34 (92%) experienced an improvement of GLILD-related parameters. 27 patients (73%) experienced sustained remission, whereas nine patients (24%) relapsed after a median of 3.2 months. Of those relapsing, two patients died of septicemia and respiratory failure, respectively.[49]

Table 4: Studies reporting treatment of GLILD in antibody deficiencies with combination therapy.
**In the paper by Verbsky et al. (2020), the total number of patients included are 39, the total number of patients treated with combination chemotherapy were 27.*

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Chase et al., 2013	Prospective follow-up cohort study	Seven patients with CVID and GLILD	Five patients received corticosteroids Rituximab at a dose of 375 mg/m2 weekly for four cycles with a four to six month interval. A total of 12-16 infusions was given Azathioprine at a dose of 1-2 mg per kg for 18 months	None	No effect of corticosteroids, combination chemotherapy led to improvement of CS and RF	Pt 1; FVC: 0.52 L increase ((% predicted increased by 9%), FEV1: 0.3 L increase ((% predicted increased by 9%), DLCO 6.89 increase ((% predicted increased by 27%). Pt 2; FVC: 0.4 L increase ((% predicted increased by 13%), FEV1: 0.11 L increase ((% predicted increased by 6%), DLCO after treatment 22.1 (98% of predicted). Pt 3; FVC: 0.11 L increase ((% predicted increased by 2%), FEV1: 0.09 L increase ((% predicted increased by 2%), DLCO 5.3 decrease ((% predicted decreased by 19%). Pt 4; FVC: 0.4 L increase ((% predicted increased by 5%), FEV1 0.4 L increase ((% predicted increased by 7%), DLCO 2.9 increase ((% predicted increased by 9%). Pt 5; FVC: 0.22 L decrease ((% predicted decreased by 4%), FEV1: 0.14 L decrease ((% predicted decreased by 2%), DLCO: 0.51 increase ((% predicted increased by 3%). Pt 6; FVC: 1.22 L increase ((% predicted increased by 33%), FEV1: 0.97 L increase ((% predicted increased by 31%), DLCO after treatment 19.00 (76% of predicted). Pt 7; FVC: 0.73 L (18% of predicted), FEV1 0.49 L (16% of predicted), DLCO 6.6 increase (20% of predicted).
Jolles et al., 2016	Case study	51-year-old woman with CVID and GLILD	Rituximab in two doses of 1g MMF for seven months	None	Improvement of PFT and RF	FVC: % predicted increased by12.5%, DLCO: % predicted increased by 10.9%
Limsuwat et al., 2018	Case study	56-year-old man with CVID and GLILD	Rituximab at a dose of 375 mg/m2 for four weeks, followed by azathioprine 200 mg/d	None	Improvement of CS, CT and PFT	FVC: 1.0 L increase (53% increase), FEV1: 0.45 L increase (46% increase)

Table 4: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Pathria et al., 2016	Case study	61-year old woman with CVID and GLILD	Rituximab at a dose of 375 mg/m2 was initiated. A total of four infusions were given Azathioprine at a dose of 0.75 per kg, which was increased to 1.5 mg per kg after two months	None	Improvement of CS and RF	Not mentioned
Routes and Verbsky, 2017	Case study	17-year old girl with CVID and GLILD	Corticosteroids for other auto-immune manifestations Rituximab and azathioprine (dose not mentioned)	None	Improvement of PFT & RF	Not mentioned
Verbsky et al., 2020	Retrospective cohort study	37 patients with CVID and GLILD	One patient received glucocorticoids prior to combination chemotherapy (dose not mentioned) Rituximab at a dose of 375 mg/m2 weekly for four cycles with a four to six-month interval. A total of 16 infusions was given Azathioprine at a dose of 1–2 mg per kg daily or MMF at a dose of 250–1000 mg twice daily for a median of 16 months		Glucocorticoids had no effect. Improvement of RF in 34/37 (92%) after combination chemotherapy. Remission was maintained in 27 patients, 9 had relapses after a median of 3.2 years, one patient underwent lung transplantation. Two patients eventually died, one of septicemia seven months after completion of treatment and the other of respiratory failure (not mentioned at which timepoint after treatment)	At baseline, FEV1 and FVC were normal in 16 (41%) patients, restrictive in 17 (44%), obstructive in 2 (%%) and mixed obstructive-restrictive in 4 (10%). 29 GLILD had DLCO measurements, 14 were normal (48%)*

Table 4: Continued from previous page.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Sood et al., 2018	Case study	16-year old boy with 22q.11 deletion syndrome, CVID and GLILD	Corticosteroids for other auto-immune manifestations Rituximab at a dose of 375 mg/m2 6-Mercaptopurine at a dose of 0.5 mg per kg three times weekly	None	Improvement of CS	Not mentioned
Tillman et al., 2019	Case study	13-year-old girl with CVID and GLILD	Rituximab at a dose of 375 mg/m2 weekly for four cycles Azathioprine at a dose of 50 mg once daily for 18 months	None	Improvement of CS and RF	FVC: increase of 64% of predicted FEV1: increase of 49% of predicted
Vitale et al., 2015	Case study	17-year-old boy with CVID and GLILD and intracranial lympho-proliferative lesions	High-dose corticosteroids Rituximab at a dose of 375 mg/m2 weekly for four cycles with a four to six-month interval. A total of 16 infusions was given Azathioprine at a dose of 1.7 mg per kg for 18 months	None	Corticosteroids had no effect, rituximab led to improvement of CS and RF with resolution of intracranial lesions	FVC: 0.62 L increase, FEV1: 0.54 L decrease

Abbreviations: CVID: common variable immunodeficiency; CS: clinical symptoms; DLCO: diffusing capacity; FVC: forced vital capacity, FEV1: forced expiratory volume in 1 second; GLILD: granulomatous-lymphocytic interstitial disease; MMF: mycophenolate mofetil; PFT: pulmonary function tests; RF: radiological findings.

Abatacept

CTLA-4 haploinsufficiency and LRBA deficiency result in a phenotype similar to CVID with severe immunodeficiency, lymphoproliferation and autoimmunity. In the physiological state, T lymphocyte responses are regulated by binding of the B7 ligand to CTLA-4 thus blocking T-cell activation, whereas LRBA is involved in intracellular trafficking and, among others, preserves CTLA-4 from degradation,[50,51] causing excessive immune activation. Abatacept consists of the

Fc region of immunoglobulin IgG1 fused to CTLA-4[52] and thus serves as a CTLA-4 fusion protein preventing excessive T lymphocyte proliferation in patients with CTLA-4 haploinsufficiency and LRBA deficiency.

A total of three articles described the use of abatacept for the treatment of GLILD (Table 5). Schwab and colleagues (2018) performed a longitudinal prospective cohort study in which they followed 133 patients with CTLA-4 haploinsufficiency. Of these, two patients who presented with GLILD treated with abatacept experienced improvement of both clinical symptoms and radiologic findings.[51]

Lo and colleagues (2015) reported three patients with LRBA deficiency and GLILD, who experienced significant improvements in lung function and radiological findings after treatment with abatacept.[53] Bal (2017) replicated these results, findings abatacept to be useful in the treatment of GLILD in a 12-year old boy with LRBA deficiency.[54]

Table 5: Studies reporting treatment of GLILD in PID with abatacept.

Article	Study Design	Sample	Intervention	Control	Qualitative Outcome	Quantitative Outcome
Kostel Bal et al., 2017	Case study	7 patients with LBRA deficiency, one of which had concomitant GLILD (12-year-old boy)	Abatacept at a dose of 20 mg per kg every two weeks, duration not specified	None	Improvement of RF	Not mentioned
Lo et al., 2015	Prospective follow-up cohort study	Nine patients with LBRA deficiency, three of whom also had GLILD	Corticosteroids and MMF, duration not specified Abatacept in different doses: 20 mg per kg every two weeks, 20 mg per kg every four weeks, 30 mg per kg monthly for six months	None	Disease progression despite treatment with corticosteroids and MMF Improvement in clinical symptoms, PFT and RF	Pt 1: FVC: % predicted increased by 30-40%, FEV1: % predicted increased 35%, DLCO % predicted increased by 35%. Pt 3: FVC: % predicted increased by 50% of predicted, FV1: % predicted increased by 40%, DLCO % predicted increased by 50%.
Schwab et al., 2018	Prospective follow-up cohort study	90 CTLA4 mutation carriers, of which 32 with GLILD	Abatacept was administered to 14 patients, duration not specified	43 unaffected mutation carriers	Six of the patients treated with abatacept experienced improvement of symptoms (two who had GLILD had resolution of lymphoproliferative lesions)	Not mentioned

Hematopoietic Stem Cell Transplantation

HSCT holds the promise of being a definitive treatment for GLILD as it can correct the underlying immunodeficiency and the associated GLILD instead of just alleviating GLILD related symptoms. However, it is associated with considerable risks, including Graft versus Host Disease (GvHD) and serious infections, both associated with considerable morbidity. This risk is likely higher in those with established structural lung disease.

Five studies reported on HSCT for CVID patients with associated GLILD (Table 6). Wehr (2015) followed 25 patients with CVID who underwent HSCT. Five patients had GLILD: four experienced an improvement of the CVID-related complications, one died 104 days after transplantation due to acute GvHD and infectious complications.[55] Wehr's papers also includes four patients which were discussed in Rizzi's publication in 2011.[56] Hartono (2018) published the case of a 23- year-old woman who presented with a CVID-like phenotype due to a STAT1 gain-of-function mutation and GLILD: after HSCT there was an improvement of radiologic findings.[57] Mixed outcomes were reported by Slatter (2016), Seidel (2018) and Tesch (2020).[58-60] Seidel and colleagues (2018) performed an international survey and collected information about 12 patients with CVID-like disease due to underlying LRBA deficiency (seven of whom also had GLILD), who underwent HSCT. Four patients went into partial remission, whereas three of them died.[59] Tesch (2020) published a prospective follow-up study of 76 patients with LRBA deficiency, of which 24 underwent HSCT. Of these 24 patients, 17 of the 24 patients survived and all of the seven patients with concomitant GLILD experienced an improvement of GLILD related symptoms. Two patients who did not have GLILD before HSCT, developed the disease after the procedure.[60]

Table 6: Studies reporting treatment of GLILD in antibody deficiencies with HSCT.

Conditioning: only conditioning regimens for patients with PADs were reported. [1]Flu, Mel and Ale, [2]Flu, ATG, Treo, [3]Flu, ATG , [4] Flu, ATG, Treo, Thiotepa, [5]Flu, ATG, Thiotepa, Mel, [6]Flu, ATG, Mel, [7]Flu, ATG, Thiotepa, [8]Flu, ATG, Treo, [9]Fly, ATG, Mel, [10]CP, Bu, [11]Flu, ATG, Mel, [12]Flu, ATG, Mel, [13]Flu, ATG, Treo, Thiotepa, [14]Flu, ATG, Treo, Thiotepa, [15]Flu, ATG, Treo, Thiotepa, [16]Flu, ATG, Mel, [17]Flu and Mel, [18]Bu and Flu,*

Article	Study Design	Sample	Control	Donor	Conditioning*	GVHD prophylaxis	Outcome (GLILD)	Outcome (Survival)
Hartono et al., 2018	Case study	23-year old girl with STAT1 mutation and GLILD	None	MUD	Not mentioned	Steroids	Improvement of radiological findings	Patient still alive day +522 post-transplant
Rizzi et al., 2011	Case study	One patient with CVID and GLILD	None	Patient 004: MUD	Patient 004: RIC[1]	CsA	Subjective improvement of PFT and reduction of steroids use	Patient with GLILD survived
Seidel et al., 2018	Prospective follow up cohort study	12 patients with LBRA deficiency of which seven also had GLILD	None	Patient 001: MFD Patient 002: MSD Patient 004: MUD Patient 006: MMFD Patient 008: MUD Patient 010: MUD Patient 01: MSD	Patient 001 RIC[2] Patitent 002 RIC[3] Patient 004 RIC[4] Patitent 006 RIC[5] Patient 008 RIC[6] Patient 010 RIC[7] Patient 011 RIC[8]	Not mentioned	Patients 002 and 010 with GLILD had complete remission (no symptoms and no need for medication), patient 001 with GLILD had good partial remission (some symptoms but no need for medication), patient 011 with GILD had partial remission (improvement of symptoms but still need for medication)	Overall survival was 67% (8/12). Patient 004, 006 and 008 with GLILD died three and two months post procedure
Slatter et al., 2016	Prospective follow up cohort study	Two patients with CTLA4 deficiency and GLILD	None	MUD	Not mentioned	Five patients (1, 2, 5, 6, and 8) CsA and MMF for GVHD. Three (3, 4, and 7) had CsA alone, CsA and MMF, or MTX and tacrolimus. Patient 6 had prednisolone, sirolimus, and belatacept until 8 days before transplant	Improvement of symptoms, tapering of immunosuppressive medication.	Six patients are still alive (two patients with GLILD fall in this group and are alive and well at 4 months and 4 years post-transplantation), two died of GvHD and DKA, respectively

Article	Study Design	Sample	Control	Donor	Conditioning*	GVHD prophylaxis	Outcome (GLILD)	Outcome (Survival)
Tesch et al., 2020	Prospective follow up cohort study	76 patients with LBRA deficiency of which 24 underwent HSCT and 17 had GLILD	Patients who did not undergo HSCT	Patient 001: MMUD Patient 002: MSD Patient 003: MSD Patient 004: MSD Patient 005: MFD Patient 007: MSD Patient 010: MUD Patient 014: MSD	Patient 001 RIC[9] Patient 002 MAC[10] Patient 003 RIC[11] Patient 004 RIC[12] Patient 005 RIC[13] Patient 007 RIC[14] Patient 010 RIC[15] Patient 014 RIC[16]	Not mentioned	Of the eight patients with GLILD, five are in complete remission, two are in partial remission with still some symptoms of GLILD. Of the 24 patients undergoing HSCT, two developed GLILD after the procedure	Overall survival was 70.8% (17/24)
Wehr et al., 2015	Prospective follow-up cohort	Two patients with CVID and GLILD	None	Patient 004: MUD Patient 029: MUD	Patient 004: RIC[17] Patient 028: MAC[18]	Patient 004: CsA Patient 028: CsA, sirolimus, MMF, corticosteroids	Patient 004: not mentioned Patient 028: deceased	Patient 028 died 104 days after procedure of aGvHD and infectious complications

Abbreviations: Ale: Alemtuzumab; ATG: anti-thymocyte globulin; Bu: Busulfan; CsA: Cyclosporin A; CP: cyclophosphamide; Flu: Fludarabine; MAC: myeloablative conditioning; Mel: Melphalan; MFD: matched family donor; MMFD: mismatched family donor; MMUD: mismatched unrelated donor; MSD: matched sibling donor; MUD: matched unrelated donor; RIC: reduced intensity conditioning.

Quality of Studies and Level of Evidence

All studies had an overall intermediate or high risk of bias (Table 7). This was largely due to the small sample sizes and lack of controls. Outcomes were mostly reported qualitatively, with few data about pulmonary function tests and a lack of standardized CT evaluation. The duration of follow-up was typically limited, meaning that long-term outcomes of patients remained uncertain. As far as confounders are concerned, smoking status was not always reported. Finally, genetic testing for CTLA-4 haploinsufficiency and LRBA deficiency only became available as of 2012, meaning that older articles could not make this additional distinction.

In 27 studies the level of evidence was 4, and in 12 studies the level of evidence of 3. The associated level of practice recommendations was weak in both groups.

Table 7: Quality of studies analyzing treatment for GLILD in primary antibody deficiencies

	Quality of the Study					Confounders				
Article	**Study Design**	**Controls**	**Outcome**	**Follow-up**	**Dose**	**Smoking**	**Age**	**Co-morbidities**	**Genetic testing**	**Risk of bias**
Arraya et al.	–	–	+/-	+	+	–	+	+	–	High
Ardenitz et al.	+	+	–	+	–	–	+	–	–	High
Boujaoude et al.	–	–	+	–	+	+	+	+	–	High
Boursiquot et al.	+	+	+/-	+	+/-	–	+/-	+/-	–	High
Bouvry et al.	+	+/-	–	–	–	–	+	–	–	High
Bucciol et al.	–	–	+/-	+	–	–	+	+	–	High
Ceserer et al.	–	–	+/-	+	+	–	+	–	–	High
Cha et al.	+	+/-	+/-	+	–	+	+	+	–	Intermediate
Chase et al.	+/-	–	+	+/-	+	–	+	–	+	High
Davies et al.	–	–	+	+	+	+ (non smoker)	+	+	–	Intermediate
Deya-Martinez et al.	–	–	+/-	+/-	+	– (children)	+	+	+	High
Franxman et al.	+/-	+/-	+	–	+	–	+	+	–	High
Guerrini et al.	–	–	+/-	–	–	–	+	+	–	High
Hartono et al.	–	–	+/-	+	NA	–	+	+	+	Intermediate
Jolles et al.	–	–	+/-	+	+	–	+	+	–	High
Kanathur et al.	–	–	+/-	+	+	+	+	+	–	Intermediate
Kaufman et al.	–	–	+	+/-	+	–	+	+	–	High

Table 7: Continued from previous page.

	Quality of the Study					Confounders				
Article	**Study Design**	**Controls**	**Outcome**	**Follow-up**	**Dose**	**Smoking**	**Age**	**Co-morbidities**	**Genetic testing**	**Risk of bias**
Kohler et al.	–	–	+	+	+	–	+	+	–	High
Kostel Bal et al.	–	–	+/–	–	+	–	+	+	+	High
Limsuwat et al.	–	–	+	+/–	+	+	+	+	–	Intermediate
Lo et al.	+/–	+/–	+/–	+	+	–	+	+	+	Intermediate
Maglione et al. (2014)	–	+	+/–	–	+	–	+	+	–	High
Maglione et al. (2019)	+	+	+/–	+	+	–	+	+	–	Intermediate
Ng et al.	–	–	+/–	+	+	–	+	+	–	High
Pathria et al.	–	–	+/–	–	+	+	+	+	–	High
Rizzi et al.	–	–	+/–	+	NA	–	+	+	–	High
Routes & Verbsky	–	–	+/–	–	–	–	+	+	–	High
Sacco et al.	–	–	+/–	+	+	–	+	+	–	High
Schwab et al.	–	+/–	+/–	–	–	–	+	+	+	High
Seidel et al.	+/–	–	+/–	+	NA	–	+	+	+	Intermediate
Slatter et al.	+/–	–	+/–	–	NA	–	+	+	+/–	High
Sood et al.	–	–	+/–	+/–	+	–	+	+	+	Intermediate
Tashtoush et al.	–	–	+/–	+/–	+	+ (non smoker)	+	+	–	High
Thatayatikom et al.	–	–	+/–	+	+	–	+	+	–	High
Tesch et al.	–	+	+/–	+	NA	–	+	+	+	Intermediate
Tessarin et al.	–	–	+/–	+/–	+	–	+	+	–	High
Tillman et al.	–	–	+	+	+	– (children)	+	+	–	Intermediate
Verbsky et al.	+/–	–	+	+	+	–	+	–	+	Intermediate
Vitale et al.	–	–	+	+	+	–	+	+	–	High
Wehr et al.	+	–	+/–	+/–	NA	–	+	+	–	High
Wislez et al.	–	–	+/–	–	+	+ (smoker)	+	+	–	High
Zdziarsky et al.	–	–	+/–	+	+	+ (non smoker)	+	–	–	High

DISCUSSION

To our knowledge, this is the most comprehensive systematic review analyzing treatment efficacy for GLILD in CVID. We show that there is still much uncertainty about the optimal treatment for GLILD and that more basic scientific and clinical research is needed in order to establish the best standard of care.

There are many factors influencing the choice of treatment. Apart from efficacy, risk-to-benefit ratio and patient preference, drug availability and cost may also play a role. Several studies reported that the efficacy of glucocorticoid monotherapy is limited. Other immunosuppressants were often used as second-line therapy with varying results. Rituximab monotherapy and combination chemotherapy with rituximab and azathioprine emerged as promising second-line treatments. Abatacept has been used in patients with CTLA-4 and LRBA mutations, but has not been routinely used in other patient populations as of yet. Finally, HSCT may be an option when other treatments have failed, but reported survival after HSCT in CVID has been poor.

Our findings suggest that glucocorticoids, although widely used as first line therapy, failed to induce remission in 57% (17 individuals) of patients using glucocorticoids. [16-22,29] Treatment with glucocorticoids led to a partial response in 13% (four individuals) and failed to maintain remission in 7% (two individuals) of patients.[16,23] There are, however, also literature reports about the positive effects of glucocorticoids.[24-27] 23% (seven individuals) of all patients using glucocorticoids had resolution of symptoms. It is currently unclear how much reporting bias has occurred in the reports describing the use of for example glucocorticoids for treatment of GLILD. Based on current knowledge, it remains unclear how the benefits of glucocorticoids in some patients may weigh against the side-effects of long-term treatment.

With respect to the category of the (biological) DMARDs, MMF, azathioprine, cyclosporine, sirolimus and infliximab have demonstrated efficacy in single case reports. Yet, because of the anecdotal nature of the studies and the relatively small patient populations they were described in, there is insufficient evidence to make definitive statements. While a previous survey has shown that most physicians agree on the implementation of azathioprine and MMF, there is no consensus as far as other (biological) DMARDs are concerned.[9]

We found that rituximab monotherapy was effective in treating GLILD in most cases, although relapses did occur after B cell reconstitution.[10,32] Combination chemotherapy with rituximab and azathioprine is another potential treatment regimen in patients with CVID and GLILD. Our collected data show that this combination of drugs was effective at inducing remission in all cases, even where other therapies had failed.[18,21,22] However, there are also indications that upon prolonged follow-up, relapses may occur.[10,49] The findings on rituximab are in line with published literature which indicates both rituximab and rituximab-based chemotherapy are effective treatments for GLILD in CVID.[9] The current literature does not allow to determine whether rituximab monotherapy is superior, equally effective or inferior to rituximab-based combination chemotherapy.

Abatacept is often implemented in the treatment of GLILD in patients with CTLA-4 haploinsufficiency and LRBA deficiency. Results were promising as the drug was effective in most reported cases. Although abatacept is mostly implemented for the treatment of patients with CTLA-4 or LRBA related diseases, it would be interesting to see whether it could be of benefit in other GLILD patient populations as well.

HSCT is a potentially curative treatment for immunodeficiencies and GLILD, yet is associated with the risk of serious complications. Our results show that when successfully carried out, HSCT does indeed lead to resolution of GLILD symptoms in most cases. One exception were two patients in the study by Tesch et al. (2020), who developed GLILD after HSCT.[60] On the other hand, the reported mortality rate was still relatively high compared to overall survival of patients transplanted for other types of PID. While for patients with CVID and GLILD the survival after HSCT varied between 48% and 70%, in PIDs in general it approaches 90%.[61] Furthermore, the procedure of HSCT encompasses immunosuppression as a result of the conditioning and replacement of hematopoietic stem cells, and it is as yet not fully proven which of these two components is responsible for the reduction of GLILD activity after HSCT. There are many factors influencing transplantation outcome, including HLA matching, severity of pre-existing lung disease, infections and the presence of active inflammation in other organs which can make transplant more hazardous. Bone-marrow microenvironment, that is, the complex interplay of local and systemic factors driving and influencing stem cell development, has recently emerged as a potential contributor to the success or failure of HSCT. As pointed out by Troilo and colleagues (2020), approximately half of patients with CVID undergoing HSCT experience incomplete B-cell reconstitution. By studying development and maturation of B-cells of immunodeficient patients

with different genetic mutations in vitro, the researchers found that patients with a non-supportive bone-marrow niche may not allow for adequate immune cell reconstitution and may have worse outcomes.[62] These findings may help in in the prediction of which CVID patients with GLILD could benefit from HSCT.

Furthermore, our study did not find clear differences in treatment responses between children (27 individuals) and adults (228) with GLILD. While mortality is higher in patients with pediatric-onset disease almost all literature reports of children with GLILD showed a positive response to treatment.[63] However, in order to make a clear statement about the prognosis of pediatric-onset GLILD, long-term follow-up data would be required.

Strengths & limitations

This is the first review that comprehensively summarizes all peer-reviewed data about the treatment of GLILD in CVID. A systematic approach was implemented according to the internationally recognized PRISMA guidelines that aimed at identifying all existing literature on the treatment of GLILD in CVID. Two databases were searched and, in order to reduce the risk of bias, the screening process was carried out by two independent blinded researchers.

Despite efforts to minimize weaknesses, several limitations need discussion. First of all, there might be bias intrinsic to the published studies. Glucocorticoids are considered first-line treatment for GLILD,[9] which could mean that their efficacy is taken for granted and successfully treated patients are under-reported.

Further, the definition of GLILD used throughout this paper may have some limitations. Even though we strictly adhered to the internationally recognized definition of GLILD used by the British Lung Foundation/United Kingdom Primary Immunodeficiency Network, we must acknowledge that GLILD is a spectrum of symptoms and manifestations and that the impact on daily life and response to treatment may differ accordingly. Hence, there is a certain degree of interindividual variation that is difficult to quantify in the absence of detailed and objective information, such as standard radiological scores and pulmonary function tests.

Moreover, we excluded several case reports describing patients with CVID and granulomatous disease, often classified as sarcoidosis, not fulfilling the current GLILD criteria. However, some of these patients may have suffered from GLILD. Indeed there are several case reports describing patients who were misdiagnosed

with sarcoidosis and who were frequently unresponsive to glucocorticoid monotherapy, similarly to the results described in this review.[64-66]

Moreover, treatment regimens were strictly defined to enable comparison of the effects of different types of monotherapy. In addition, strict criteria for evaluation of remission of GLILD were formulated. Because of this, small positive effects of treatment might have been underreported in this study.

Finally, long-term effects of medication are seldom mentioned, including the risk of infection linked to the prolonged use of immunosuppressants. This could either mean that the added effect of immunosuppressants in already immunocompromised individuals is negligible or that there is some degree of reporting bias at play. Similarly, little to no side-effects were mentioned in the analyzed literature. However, glucocorticoids are unsuitable long-term therapy candidates because of detrimental effects on metabolism, bone density, growth and behavior. As mentioned previously, the quality of the evidence was relatively low, because none of the included studies had an experimental set-up. The choice of outcome measures was heterogeneous, and often only qualitative assessments were made, thus preventing meta-analysis. Possible confounders were rarely mentioned in the reviewed literature. Hence, it was difficult to make any final recommendations for clinical practice based on the available literature.

Future directions

Understanding the cause of GLILD is critical in finding a cure for this disease. About 10-20% of patients with CVID[7] develop GLILD, which suggests that the complication is brought on by a combination of (epi-) genetic and/or environmental factors rather than a single cause. It could be postulated that individuals with GLILD are a specific subset of the patient population with CVID, with a susceptibility for lymphoproliferation. Reverse thinking by translating from the bench back to hypothesis formulation can help assemble a workable theoretical framework. If, as is currently thought, GLILD is a form of immune dysregulation,[67] there are potentially two important players, namely T-cells and B-cells.

The efficacy of second-line immunosuppressants that selectively target T-cells suggest they have an important role in the pathogenesis of GLILD. On the other hand, the successful use of rituximab in the treatment of the disease supports the idea that B-cells may be important effector cells, either initiating or maintaining inflammation in GLILD. A combined role of T- and B-lymphocytes has also been

suggested:[18] superior efficacy of the combination of azathioprine and rituximab compared to rituximab monotherapy would plead in favor of this hypothesis.

However, fundamental research into the pathophysiology of GLILD is needed to corroborate any of the above-mentioned hypotheses. In patients in whom monogenetic defects are identified, personalized medicine with individualized treatment strategies could be devised. Histopathological analysis, where available, may support this. Abatacept in CTLA-4 haploinsufficiency and LRBA deficiency is a good example of how personalized medicine is already being implemented in clinical practice.

In order to improve patient care and treatment of GLILD, it is important to screen for the condition,[9] and define the best standard of treatment. RCTs are still lacking, because, due to the low incidence of GLILD, it is difficult to recruit sufficient numbers of participants. However, a combined effort by international consortium of medical centers, could allow for standardized data collection on a much larger scale, including pulmonary function tests and a uniform radiographic high-resolution CT scan score. Indeed, studies such as STILPAD are on-going and will inform on this. Until then, uniform standardized reporting on GLILD is crucial. Based on previous literature, this should at least include information on how the GLILD diagnosis was made, dosage and interval of the intervention, treatment-associated side effects (both short- and long-term), pre- and post-treatment CT scores using a universal scoring method, pulmonary function tests including carbon-monoxide diffusion and lymphocyte phenotyping data, ideally using validated tools. Results could provide scientific backup for current treatment strategies and help create new, evidence-based treatment protocols.

CONCLUSION

Based on this systematic review of the current literature, which was often of low quality with a high risk of bias, it is impossible to define which therapeutic option is optimal in treating GLILD in CVID.

Corticosteroid monotherapy seems suboptimal for many patients, rituximab monotherapy and combination chemotherapy with rituximab and azathioprine were effective in most reported cases. The use of abatacept has so far been only implemented as therapy for patients with pathogenic CTLA-4 and LRBA mutations. HSCT is the only curative treatment for GLILD, yet not free of risks. While much is

left open and uncertain, what has become most evident throughout this review is that there remain many critical knowledge gaps concerning treatment of GLILD. Etiology and optimal treatment for the disease are questions that require urgent answers, as they may lead to better and more specific treatment regimens. In the future, larger well-designed studies evaluating therapeutic strategies should be carried out, with uniform quantitative outcomes.

APPENDIX: SEARCH STRING

Population: patients with PID and GLILD
Intervention: treatment (pharmacological and/or stem cell transplantation)
Control: no therapy or placebo
Outcome: clinical symptoms, pulmonary function tests, radiologic findings, mortality

PubMed

"common variable immunodeficiency"[MeSH] OR CVID [Title/Abstract] OR common variable immunodeficiency [Title/Abstract] OR primary immunodeficiency [Title/Abstract] OR GLILD [Title/Abstract] OR antibody deficiency [Title/Abstract] OR granulomatous lymphocytic interstitial lung disease [Title/Abstract] OR granulomatous disease[Title/Abstract] OR interstitial lung disease [Title/Abstract] OR ILD [Title/Abstract] OR granulomatous lung disease [Title/Abstract] OR lymphocytic interstitial pneumonitis [Title/Abstract] OR lymphoid interstitial pneumonitis [Title/Abstract] OR LIP [Title/Abstract]

AND "hematopoietic stem cell transplantation"[MeSH] OR hematopoietic stem cell transplantation[Title/Abstract] OR HSCT[Title/Abstract] OR stem cell transplantation[Title/Abstract] OR SCT[Title/Abstract] OR "abatacept"[MeSH] OR abatacept[Title/Abstract] OR corticosteroid*[Title/Abstract] OR prednisone[Title/Abstract] OR methotrexate[Title/Abstract] OR "mycophenolic acid"[MeSH] OR "mycophenolic acid" [Title/Abstract] OR mycophenolate mofetil[Title/Abstract] OR rituximab[Title/Abstract] OR "azathioprine"[MeSH] OR azathioprine[Title/Abstract] OR immunosuppressant[Title/Abstract] OR immunomodulator[Title/Abstract]

EMBASE

'common variable immunodeficiency'/exp OR 'common variable immodeficiency':ab,ti,kw OR CVID:ab,ti,kw OR 'primary immunodeficienc*':ab,ti,kw OR 'antibody deficiency':ab,ti,kw OR GLILD:ab,ti,kw OR 'granulomatous lymphocytic interstitial lung disease'/exp OR 'granulomatous lymphocytic interstitial lung disease':ab,ti,kw OR ILD:ab,ti,kw OR 'granulomatous lung disease':ti,ab,kw OR 'interstitial lung disease':ab,ti,kw OR 'lymphocytic interstitial pneumonia':ti,ab,kw OR 'lymphocytic interstitial pneumonitis':ti,ab,kw OR 'lymphoid interstitial pneumonitis':ti,ab,kw

AND 'stem cell transplantation'/exp OR 'stem cell transplantation':ti,ab,kw OR 'hematopoietic stem cell transplantation':ti,ab,kw OR abatacept/exp OR abatacept:ab,ti,kw OR corticosteroid/exp OR corticosteroid:ab,ti,kw OR prednisone:ab,ti,kw OR 'mycophenolic acid'/exp OR 'mycophenolic acid':ti,ab,kw OR 'mycophenolate mofetil'/exp OR 'mycophenolate mofetil':ti,ab,kw OR methotrexate/exp OR methotrexate:ab,ti,kw OR immunosuppressant:ti,ab,kw OR immunomodulator:ab,ti,kw

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Conflict of Interest

John Hurst and Klaus Warnatz co-chair the European Respiratory Society-funded e-GLILDnet Clinical Research Collaboration which is a collaboration with ESID (the European Society for Immunodeficiencies). The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Olivia Lamers and Bas Smits created the search string, selected the articles included in the review, wrote the paper and created the tables. Joris van Montfrans chose the review topic, and guided the research and writing process. Janneke van de Wijgert gave advise about the methodology and reviewed the final text. Charlotte Cunningham-Rundles and Hsi-en Ho provided additional raw data which was included in the review. Virgil Dalm, Godelieve de Bree, John Hurst, Hsi-en Ho, Hanna IJspeert, Helen Leavis, Suzanne Terheggen – Lagro, Sabine Prevaes, Alex Robinson, Astrid van Stigt, Annick van de Ven and Klaus Warnatz gave advise during the synthesis of the results, commented on the draft papers and reviewed the final text.

Funding

Financial support for this publication was provided by the Louise Vehmeijer Foundation, "e-GLILDnet" and the European Respiratory Society.

CHAPTER 10

The effectivity and toxicity of corticosteroids as first line treatment for GLILD

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ABSTRACT

Background: Granulomatous and lymphocytic interstitial lung disease (gl-ILD) is a major cause of morbidity and mortality among patients with common variable immunodeficiency. Corticosteroids are recommended as first-line treatment for gl-ILD, but evidence for their efficacy is lacking.

Objective: We analyzed the effect of high-dose corticosteroids (≥0.3mg/kg prednisone equivalent) on gl-ILD, measured by high-resolution computed tomography (HRCT) scans, pulmonary function test (PFT) results.

Methods: Patients who had received high-dose corticosteroids but no other immunosuppressive therapy at the time and who underwent repeated HRCT scanning or PFT during the retrospective and/or prospective phase of the STILPAD study (n=56) were included in the analysis. Patients without any immunosuppressive treatment were selected as controls (n=23). HRCT scans were blinded, randomized and scored using the Hartman score. Differences between the baseline and follow up HRCT scans and PFT were analyzed.

Results: Treatment with high-dose corticosteroids significantly improved HRCT scores and forced vital capacity. Carbon monoxide diffusion capacity significantly improved in both groups. 13/18 patients, for whom extended follow-up data was available, achieved a long-term, maintenance therapy independent remission. All patients with relapse were re-treated with corticosteroids, but only 1/5 responded. Two opportunistic infections were found in the corticosteroid treatment group, while overall infection rate was similar between cohorts.

Conclusion: Induction therapy with high-dose corticosteroids improved HRCT scans and PFT results of patients with gl-ILD and achieved long-term remission in 42% of patients. It was not associated with major side-effects. Low dose maintenance therapy provided no benefit and efficacy was poor in relapsing disease.

Clinical Implications

High-dose corticosteroids appear to be an effective, safe, widely available first-line treatment for gl-ILD. However, some patients require more effective first-line therapies and long-term and repetitive steroid therapy seem unfavorable.

Capsule Summary

gl-ILD is a major complication of common variable immunodeficiency. Corticosteroids are propagated as first-line treatment, but have been scarcely investigated. This study provides the first evidence for the safety and efficacy of corticosteroids in gl-ILD.

Key words

Granulomatous and lymphocytic interstitial lung disease; gl-ILD; CVID; immune dysregulation; observational trial; corticosteroids; Hartmann Score; pulmonary function tests; quality of life;

ABBREVIATIONS

BLF: British lung foundation
CRQ-SAS: chronic respiratory questionnaire self-administered standardized
CVID: common variable immunodeficiency
$DLCO_{SB}$: diffusion capacity of the lung for carbon monoxide in a single breath
FEV1: forced expiratory volume in one minute
FVC: forced vital capacity
gl-ILD: granulomatous lymphocytic interstitial lung disease
HRCT: high resolution computed tomography
IgM: immunoglobulin M
PCA: principal component analysis
PFT: pulmonary functions tests
QoL: quality of life
SF-36: 36-item short form health survey
SGRQ: St George's respiratory questionnaire
sIL-2R: soluble interleukin 2 receptor
TLC: total lung capacity
UKPIN: UK primary immunodeficiency network

INTRODUCTION

Common variable immunodeficiency (CVID) is associated with clinical manifestations of immune dysregulation in more than 30% of patients. [1,2] These comprise generalized lymphoproliferation, auto-immune disease, especially targeted towards blood cells, and inflammatory organ involvement, including celiac-like enteropathy and granulomatous lymphocytic interstitial lung disease (gl-ILD).[3-6] These immune dysregulatory manifestations often require additional immunosuppressive therapies and have become a major cause of morbidity and mortality for CVID patients.[7]

As most patients present with more than one complication of immune dysregulation, these complications are considered part of a systemic immune disorder. Thus, in several cohort studies, gl-ILD has been associated with systemic granulomatous disease affecting other organs, splenomegaly, diffuse lymphoproliferation and autoimmune cytopenia. [8-11]

In CVID patients, a number of potential laboratory markers have been associated with gl-ILD manifestation or activity including sparse switched memory B cells, an expansion of $CD21^{low}$ B cells and higher IgM and BAFF serum levels during active disease, all indicating B-cell overactivation. [8, 9,12,13] Additionally, CVID patients with gl-ILD show increased serum levels of soluble IL-2 receptor (sIL-2R),[14, 15] CD6, and CD28 indicating T-cell activation. Thus, gl-ILD seems to involve a broad activation of both the adaptive and the innate immune system.[16,17]

The histology of gl-ILD includes components of granulomatous inflammation, lymphocytic interstitial pneumonitis, bronchiolitis and cryptogenic organizing pneumonia.[18-20] The lymphocytic infiltrates consist of both T and B cells, and in some biopsies lymphocytic aggregates and even tertiary germinal centers can be observed.[13, 18] Until now, no infectious trigger has been convincingly identified.

In regard to treatment of gl-ILD, no standardized treatment protocol currently exists. Corticosteroid monotherapy is often used as first-line treatment for gl-ILD and has been proposed as such in a consensus statement of the British lung foundation (BLF).[21] However, a recent systematic review found little evidence for the efficacy of corticosteroids, partly due to a very limited number of reported patients treated with corticosteroids. [22,23] In a recent survey on research prioritization in gl-ILD, studies analyzing the short-term and long-term efficacy and associated risk of corticosteroids were given top priority by both clinicians and patients. [24]

Therefore, we used retrospective and prospective data of the observational multicenter STILPAD study to systematically describe the effect of corticosteroid monotherapy on gl-ILD. We investigated the efficacy and safety of induction therapy with high-dose corticosteroids (≥0.3mg/kg prednisone equivalent) and estimated response and relapse rate after therapy.

METHODS

Type of study

The dataset for this analysis was derived from STILPAD, a non-interventional, multicenter European study (DRKS-ID: DRKS00000799) of CVID patients with gl-ILD. In this study, 144 participants were recruited for a retrospective and prospective observational analysis. Retrospective documentation started at the first event of either gl-ILD diagnosis, pulmonary functions tests (PFT) or chest high resolution computed tomography (HRCT). The prospective observation was started in June 2012 and was performed over a period of up to five years (last patient out January 2018).

Study Population

The inclusion criteria for STILPAD were:

- Diagnosis of CVID according to the 1999 ESID criteria
- Chest HRCT positive for nodules, lines or ground-glass signs compatible with interstitial lung disease or granuloma
- Age ≥ 18 years
- Written informed consent

Patients were eligible for this study if:

- Inclusion criteria for the corticosteroid group or the control group were met (see below)
- Study data was recorded during the STILPAD study
- Data on primary or secondary outcomes was reported

Corticosteroid group:

- At least one course of ≥0.3mg/kg and ≥30mg prednisone equivalent dose.
- No other immunosuppressive therapy 5 years prior to corticosteroid therapy
- No other immunosuppressive therapy prior to response evaluation

- CT, PFT, biomarker or quality of life data was recorded within one year prior to treatment initiation (T0) and within 24 months after treatment initiation (T1).

OR

Prospective data on infections was gathered during treatment.

OR

Medical history containing steroid related complications was reported

Control group:

- No immunosuppressive therapy during the last 5 years before or during STILPAD
- CT, PFT, biomarker or quality of life data was recorded 12-24 months (T0-T1) apart.

OR

Prospective data on infections was gathered.

OR

Medical history containing steroid related complications was reported

Parameters collected during STILPAD, relevant to this study are listed in Electronic Repository Table 1.

Study endpoints

The primary endpoint of this study was the effect of corticosteroids within 24 months after initiation on four predefined gI-ILD-related clinical parameters; the HRCT based ILD score and the ground-glass sub score, forced vital capacity (FVC) and the diffusion capacity of the lung for carbon monoxide in a single breath ($DLCO_{SB}$).

Moreover, this study analyzed response and relapse occurrence within the corticosteroid group. HRCT response was quantified as a relevant improvement of the ILD score (5 points) and PFT response was evaluated using the American

Thoracic Society/European Respiratory Society criteria (10% improvement of FVC or 15% improvement of $DLCO_{SB}$) within two years after corticosteroids were started.[15] Relapse was defined as a clinically relevant deterioration of the ILD score, the FVC or the $DLCO_{SB}$ during follow-up.

Additionally, this study assessed the safety of corticosteroid therapy in gl-ILD patients, through the evaluation of infections and corticosteroid related complications.

Finally, we estimated the effect of corticosteroids on secondary parameters including the other HRCT scores, physiological, and laboratory parameters (Electronic Repository Table 1) and we analyzed co-correlation between these parameters and the primary endpoint parameters. In addition, we evaluated quality of life (QoL using the 36-Item Short Form Health Survey (SF-36) for general functional health and well-being, as well as the CRQ-SAS (Chronic Respiratory Questionnaire Self-Administered Standardized) and SGRQ (St George's Respiratory Questionnaire).[25,26,27]

Radiographic evaluation

All HRCT scans were scored centrally by two blinded, trained investigators using the Hartmann method.[28,29] With this method, each lobe was scored separately on a scale of 0 to 3 for a total of 26 items, including ILD parameters. ILD parameters consisted of ground glass changes, reticulation with and without distortion and the number, contour and size of nodules.

Pulmonary function evaluation

Pulmonary function evaluation consisted of TLC, FVC, FEV1, $DLCO_{SB}$, resting O_2, and pO_2 and were measured at regular intervals specified in E Repository Table 1.

Statistics

Medians were analyzed with Mann-Whitney U tests and proportions with N-1 Chi Square tests. Medians of longitudinal parameters were analyzed using paired two-sided Wilcoxon-Signed Rank tests. Relapse occurrence was analyzed using Cox regression. Significance was reached with $p<0.05$. To prevent multiple testing, QoL assessments were first analyzed with principal component analysis (PCA). R Studio version 1.2.5019 was used to analyze the data.

RESULTS

Study Population

We included 79 patients from the STILPAD study, 56 in the corticosteroid group and 23 in the control group (Electronic Repository Figure 1) and further specified the treatment regimens of the corticosteroid group (Electronic Repository Table 2).

In the STILPAD study, radiographic signs of gl-ILD were sufficient for a gl-ILD diagnosis. To evaluate the likelihood of gl-ILD in this study we further classified the likelihood of gl-ILD diagnosis. The gl-ILD diagnosis was classified as probable if patients either had histological confirmation or a gl-ILD probability score >50% and radiographic signs of gl-ILD.[9] The gl-ILD diagnosis was classified as possible if patients only had radiographic signs of gl-ILD.

Baseline characteristics were compared between the steroid and control group (Table 1). This revealed a significantly worse FVC and ILD score and a trend towards a worse $DLCO_{SB}$ at baseline in the corticosteroid group.

Table 1: gl-ILD patients treated with corticosteroids showed a trend towards worse baseline primary endpoint parameters and longer gl-ILD duration. *Baseline parameters of patients without or steroid treatment. The gl-ILD probability was calculated according to Cinetto et al. Means were compared with Mann-Whitney U tests, percentages were compared with N-1 Chi-squared tests.*

	Control Group	Corticosteroid Group	p-value
N	23	56	
General Parameters			
Age (years)	47 (20 – 73)	45.5 (19 – 77)	0.61
Females (%)	56.5	66.1	0.42
Genetic Diagnosis (%)	9.1	18.9	0.44
Probable gl-ILD	82.6	84.6	0.83
Splenectomy (%)	8.7	12.5	0.63
Alive (%)	91.3	87.5	0.63
Pulmonary Status			
Former Smokers (%)	26.1	32.1	0.52
Current Smokers (%)	13.0	5.4	0.24
GLILD duration (years)	6.4 (1.3 – 22.4)	7.8 (4.1 – 32.5)	0.11
Asthma (%)	9.1	7.1	0.77
Bronchiectasis (%)	43.5	46.4	0.81
COPD (%)	0	7.1	0.18
EUROclass			
B^- (%)	4.3	2.2	0.63
smB^-21^{lo} (%)	47.8	46.7	0.93

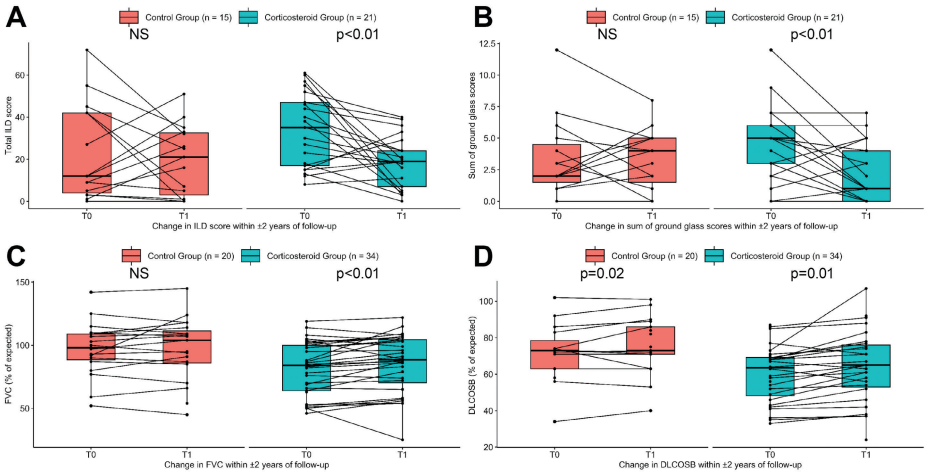
Table 1: Continued from previous page.

	Control Group	Corticosteroid Group	p-value
smB^{-}21^{normal} (%)	21.7	24.4	0.81
smB^{+}21^{lo} (%)	8.7	22.2	0.17
smB^{+}21^{normal}(%)	13.0	4.4	0.20
Other Immune Dysregulation			
Auto-immune cytopenias (%)	34.8	55.4	0.09
Other auto-immune disease (%)	0	19.6	0.02
Enteropathy/IBD (%)	60.9	60.7	0.99
Lymphoproliferation (%)	95.7	82.1	0.28
Hematologic Malignancies (%)	4.3	10.7	0.37
Solid Malignancies (%)	0	3.6	0.36
Baseline primary endpoint parameters			
ILD-score	12 (0 – 71)	32.5 (6 – 61)	0.04
Ground glass sum score	2 (0 – 12)	4.5 (0 – 12)	0.15
FVC (% of expected)	98 (52 – 142)	83.5 (46 – 119)	0.01
$DLCO_{SB}$ (% of expected)	73 (34 – 102)	64 (33 – 87)	0.03
Included in primary outcome analysis			
CT (n)	15	21	
PFT (n)	20	34	
Included in safety analysis			
Infections (n)	23	36	
Complications (n)	22	38	

Primary Endpoint

We evaluated the primary endpoint in the corticosteroid group (n=39/56) and the control group (n=20/23) for patients that had reported primary endpoint data available (Electronic Repository Figure 1). HRCT scores decreased significantly within 24 months after treatment initiation (ILD score: -11 [-54 - 18] and ground glass subscore: -2 [-7– 3], Figure 1). PFT parameters improved significantly within 24 months after treatment initiation (FVC: 4% [-25% – 19%] and $DLCO_{SB}$: 3.7% [-7% - 24%], Figure 1); however, $DLCO_{SB}$ improved similarly in the control group (3.7% [-11% - 12%]). Treatment was initiated during the retrospective phase of this study for 20/39 patients. The median prednisone equivalent dose was 0.67 mg/kg (0.31 – 1.23) and the median treatment duration was 1.5 years (1 month – 10.5 years). gl-ILD diagnosis was probable in 37/39 patients in the corticosteroid group and in 16/20 patients in the control group. Similar response was seen in a subgroup analysis that only contained patients with a probable diagnosis of gl-ILD.

Figure 1: Corticosteroid treatment leads to significant improvement of ILD related HRCT scores and PFT parameters. A-B *The ILD score and Ground Glass sum score measured at baseline and within two years of follow-up for the control group and corticosteroid treated patients. Total ILD was calculated by adding up the Nodules, Ground Glass and Reticulation sub scores of all lung fields. Patients in the corticosteroid group show a significant reduction of both scores.* ***C-D*** *Forced vital capacity (FVC) and diffusion capacity of the lung for carbon monoxide in a single breath (DLCO) at baseline and within two years of follow-up. Patients in the corticosteroid group show a significant improvement of both FVC and DLCO while the control group only showed significant improvement of DLCO*



These results showed that corticosteroid treatment improved gl-ILD related parameters within 24 months. In order to see whether corticosteroid treatment could improve gl-ILD related parameters within a shorter timeframe, we performed a subgroup analysis that only included patients whose primary endpoints were evaluated within 12 months. This subgroup analysis showed similar results, suggesting that corticosteroid treatment could also improve gl-ILD related parameters within 12 months (Electronic Repository Figure 2).

Response & Relapse Free Survival

Next, we classified patients in the corticosteroid group into responders and non-responders using our previously defined response criteria.[15] We found that 26/39 patients responded (67%). Combined PFT and HRCT data was available for 16 patients. The PFT of two patients improved, while their HRCT did not. This inconsistency could be explained by the fact that in both cases HRCT was performed at a later date and the patients had relapsed according to PFT from this later date. The HRCT of six patients improved while their PFT remained stable and the HRCT of one patient improved while his PFT deteriorated.

We compared the distribution of baseline parameters, sIL-2R, neopterin, and IgM among responders and non-responders and found a higher frequency of $CD21^{low}$ expressing B cells (87.5% vs 44%, p=0.01) among responders, along with more nodules (33.5 vs 11, p=0.01), and more ground-glass lesions (5.0 vs 2.5, p=0.03) on HRCT. These HRCT aberrancies resulted in higher ILD scores (39 vs 15, p=0.03) and more reticulation (6 vs 2 p=0.05).

We also investigated the relation between the baseline primary endpoint parameters, corticosteroid dosage, and response, but found that only higher baseline ILD scores related to an increased response ($r = 0.76$, $p<0.0001$, Electronic Repository Figure 3). Moreover, an ILD score of >18 identified responders to corticosteroids with 94% sensitivity and 100% specificity and an AUC of 0.97.

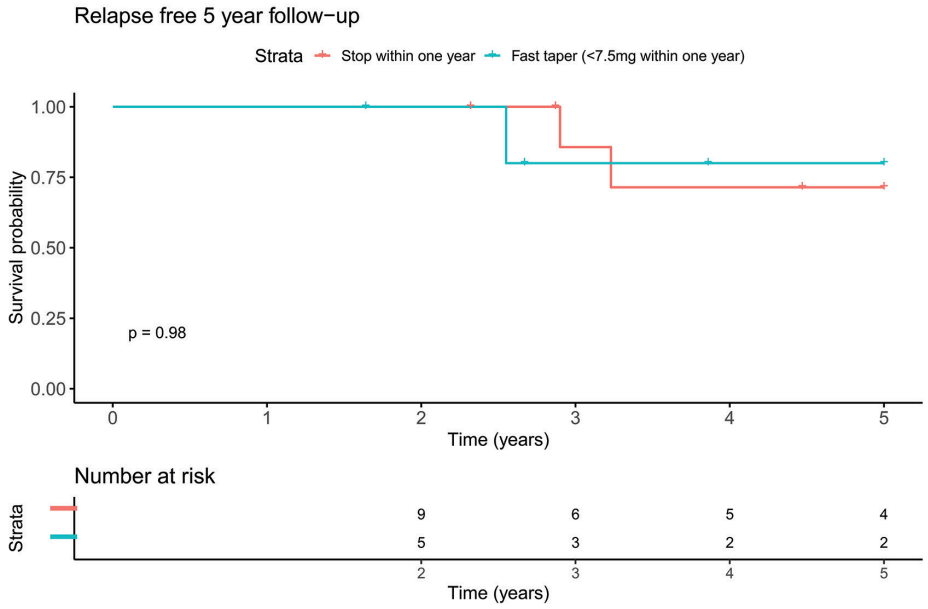
Then we used the same criteria to classify patients who had responded to corticosteroid treatment into patients who had relapsed and patients that had achieved prolonged remission. Follow-up data was available for 18 responders.[15] A prolonged remission of at least two years was achieved in 13/18 responders. Prolonged remission was associated with worse baseline PFT parameters (FVC: 72% vs 88%, p=0.055, DLCOSB: 57% vs 69%, p=0.04). Relapse was seen in 5/18 responders. Relapse was treated with additional courses of corticosteroids, but prolonged remission was achieved in only 1/5 patients.

Finally, we compared the relapse rate of patients that received maintenance corticosteroid therapy and patients that were weaned off corticosteroids completely. Three responders were tapered very slowly and were excluded from this analysis. The two groups relapsed at a similar rate (Figure 2), after a median follow-up of 3.9 years (0.27-16.69).

Infections & Steroid Related Complications

We investigated whether corticosteroid treatment was associated with an increase in infections. To determine this, we analyzed the occurrence of infections before and after the start of corticosteroids (Electronic Repository Figure 4A). We also compared the occurrence of infections in the corticosteroid group and the control group (Table 2) and we analyzed the occurrence of infections over time (Electronic Repository Figure 4B). No significant differences were found, suggesting that corticosteroid treatment is not associated with an increase in infections.

Figure 2: Relapse rates are comparable between patients with and without steroid maintenance therapy. *Response and relapse were classified as a relevant change in HRCT scores and/or PFT parameters (ILD score: ±5 points, FVC: 10%, DLCOSB: 15%). Relapse was analyzed among 15 patients for whom steroids were either stopped within one year or tapered to a dose <7.5mg, using a Cox-proportional Hazard model. Censored patients are marked with a +. No significant difference was found between the two groups (p=0.54)*



However, two opportunistic infections were observed during maintenance corticosteroid treatment. One patient suffered from a severe opportunistic fungal infection and one from a complicated zoster infection. These patients were not distinct in their original CD4 T cell count and previous infection history. Both recovered fully after adequate therapy.

We also compared the occurrence of other steroid-related complications in the patient histories and found that patients from the corticosteroid group potentially had more skeletal complications. Skeletal complications consisted of osteoporosis (n=7), compression fractures (n=2) and an osteoporotic fracture (n=1). Only one case of osteoporosis was reported in the control group. Fractures only occurred in patients under maintenance corticosteroid treatment.

Table 2: Corticosteroid treated patients do not suffer from more respiratory tract infections. *The mean follow-up time between the corticosteroid treated patients and control group significantly differed, hence the total available years of follow-up and the number (#) of infections were used to calculate the mean number of infections per year, this was also further specified per affected organ. Moreover, proportion of infections that required antibiotic treatment, proportion of infections that had a complicated clinical course requiring hospitalization or prolonged treatment were comparedusing Mann Whitney U tests or N-1 Chi squared tests, as appropriate.*

Characteristic	Control group	Corticosteroid group	p-value
N	23	36	
Median follow-up time (yrs)	5.0	1.39	<0.001
Years of follow-up (n)	124	81	
Antibiotics required (%)	90.1	86.3	0.30
Complication (%)	21.9	19.6	0.61
Infections (n)	151	168	
All Infections (median/p/y)	1	1.76	0.08
Respiratory Infections (median/p/y)	0.83	1.04	0.66
- Upper Tract (median/p/y)	0.4	0	0.23
- Lower Tract (median/p/y)	0.43	0.57	0.94
Sepsis (median/p/y)	0	0	0.71
Soft Tissue Infections (median/p/y)	0	0	0.71
GI Infections (median/p/y)	0	0	0.30
Urogenital Infections (median/p/y)	0	0	0.16
Opportunistic Infections (n)	0	3	
Opportunistic Infections (median/p/y)	0	0	0.10
Other Infections (median/p/y)	0	0	0.56

GI: Gastrointestinal, median/p/y: median/patient/year, yrs: years

Table 3: More skeletal complications were reported for corticosteroid treated patients. *Percentages of reported complications possibly related to steroids reported in the medical history were compared between corticosteroid treated patients and the control groupusing N-1 Chi squared tests.*

	Control group	Corticosteroid group	p-value
N	22	38	
Complications per patient (median)	0	0	
Any complications (%)	31.8	36.8	0.70
Skeletal (%)	4.5	23.7	0.06
Vascular (%)	13.6	16.7	0.75
Metabolic (%)	9.1	2.6	0.27
Gastrointestinal (%)	9.1	5.3	0.57
Endocrinological (%)	4.5	13.2	0.28

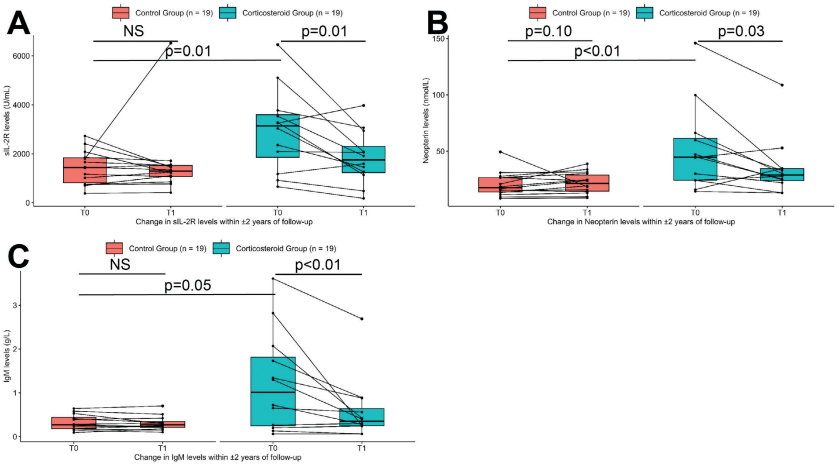
Secondary Endpoints

In regard to secondary endpoints, first, we determined if corticosteroid treatment could improve other HRCT scores (Electronic Repository Figure 5). This showed that corticosteroid treatment could reduce the nodule, reticulation and airway disease scores significantly.

Next, we evaluated if corticosteroid treatment could improve other PFT parameters, the resting oxygen saturation or pO_2 (Electronic Repository Figure 6). Here, corticosteroid treatment improved the FEV1 significantly. Moreover, the TLC significantly improved in the control group and resting pO_2 significantly improved in both groups.

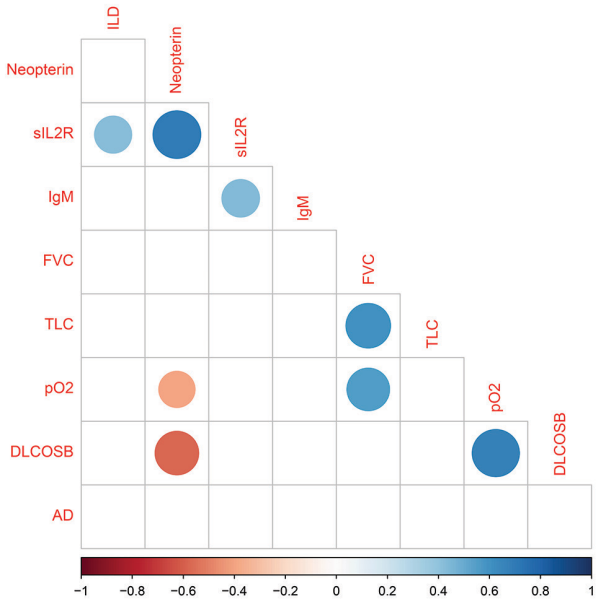
Then we explored the performance of three potential biomarkers (sIL-2R, neopterin and IgM, Figure 3). Baseline levels were significantly higher in the corticosteroid group and all three biomarkers significantly decreased after treatment.

Figure 3: Laboratory markers of active gl-ILD respond to corticosteroid treatment. *sIL-2R (**A**), neopterin (**B**) and IgM (**C**) levels were measured at baseline and within two years of follow-up for the control group and corticosteroid group. Corticosteroid treated patients showed higher sIL-2R, neopterin and IgM levels at baseline (Mann-Whitney U test) and all showed a significant reduction after corticosteroid treatment (Wilcoxon-signed rank test)*



Finally, we evaluated the relation between primary and secondary outcomes in a correlation matrix (Figure 4). This showed that higher sIL-2R levels were related to higher ILD scores ($r = 0.42$, $p < 0.01$) and higher neopterin levels were related with a worse $DLCO_{SB}$ ($r = -0.58$, $p < 0.01$), and resting pO_2 ($r = -0.4$, $p = 0.02$). Additionally, biomarker levels were intercorrelated. A correlation between the ILD score and PFTs was not found.

Figure 4: Laboratory markers correlate with gI-ILD activity. *PFT results, pO, the ILD HRCT score, sIL-2R, neopterin and IgM that were acquired in the same year were collected. Missing data was imputed using stochastic regression imputation. The correlation matrix shows correlations with a p<0.05 and shows strong correlation between sIL2R and neopterin, a weaker correlation between sIL-2R and IgM and between sIL-2R and the ILD score. Neopterin correlates strongly with DLCOInterestingly, there are no correlations between the ILD score and PFT results.*



Quality of Life Assessment

Finally, we performed a principal component analysis with oblique rotation on the 15 domain sum scores from the quality-of-life questionnaires of the control group (n=15) and corticosteroid group (n=17) to identify domains that could distinguish between these groups.

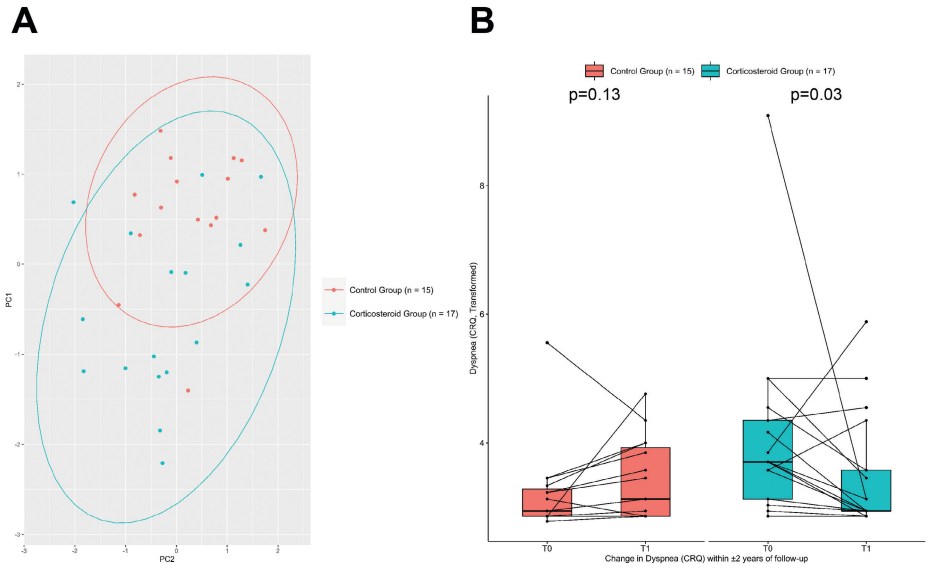
However, there were too few samples to explain 15 domain sum scores, expressed by the Kaiser-Meyer-Olkin (KMO) measure of common variance. The domain with the poorest common variance was excluded, which was the emotional well-being domain (SF-36). This resulted in acceptable PCA prerequisites, with a KMO of 0.83 and a highly significant Bartlett's test. Three principal components were able to explain most of the variance within the dataset (75%).

Cluster analysis showed that principal component 1 (PC1) and principal component 2 (PC2) could potentially distinguish between the corticosteroid group and the control group (Figure 5A). PC1 correlated to the CRQ domains and the symptoms

domain of the SGRQ. PC2 correlated to the pain and physical functioning domains of the SF-36, and the impact and activity domains of SGRQ.

This suggested that patients treated with corticosteroids scored differently on these nine domains at baseline. Therefore, baseline values were compared between the corticosteroid group and the control group. This showed that pain (p=0.02), physical functioning (p<0.01), fatigue (p=0.03), physical activity (p=0.01) and dyspnea (p=0.04) were worse in the corticosteroid group. Next, we evaluated the effect of corticosteroid treatment on the five hampered domains and found that corticosteroid treatment only improved dyspnea (Figure 5B) while the others remained stable.

Figure 5. Dyspnea is responsive to corticosteroid treatment. *Three quality of life reports (SF-36, CRQ & SGRQ) were collected at baseline and within 2 years after treatment initiation. (**A**) PCA was performed on the calculated baseline domain scores to distinguish between corticosteroid treated patients and control patients and 9 domains divided among PC1 and PC2 were found. (**B**) Of these domains dyspnea significantly improved after corticosteroid treatment (Wilcoxon-Signed rank test). CRQ, Chronic Respiratory Questionnaire.*



DISCUSSION

This multicenter retrospective and prospective observational trial is the first to quantify the efficacy and safety of corticosteroid therapy as treatment for gl-ILD. Our data demonstrates that corticosteroid treatment led to a radiographic and/or pulmonological response in 67% of patients, and did not increase reported respiratory tract infections. We also show that steroid treatment improved patient reported dyspnea.

High-dose corticosteroid treatment (≥0.3mg/kg) was effective in improving the HRCT and/or PFT in 67% of patients. HRCT findings improved more frequently than PFT. We considered an improvement of >5 points on HRCT or an improvement of >10% FVC or >15% $DLCO_{SB}$ to be a response. Response on HRCT was achieved in 76% of patients, while response on PFT was achieved in 37% of patients. This discrepancy in HRCT and PFT response has recently been shown by Verbsky *et al.,* who described the efficacy of combination therapy for gl-ILD consisting of rituximab and azathioprine or mycophenolate mofetil. [30]

The radiographic response rate (76%) to corticosteroids in this study was similar to the response rate to abatacept (71%), but lower than the response rate to combination therapy (92%).[15,30] Even if the response rates between corticosteroid and combination therapy cannot be compared directly due to different study design and radiological scoring systems, the fact that most patients treated with combination therapy had failed previous corticosteroid treatment still clearly demonstrates the superiority of the combination regime in a subgroup of patients. [30]

High-dose corticosteroid treatment not only induced short-term but also long-lived remission in the majority of responders. Remission was maintained for at least 2 years in 72% of patients with primary response for whom follow-up data was available suggesting a relevant subgroup of patients for whom corticosteroid therapy may represent not only a supportive, but sufficient first-line treatment option.

Patients that received low dose maintenance corticosteroid therapy did not have an improved remission rate compared to patients that were weaned off corticosteroids completely. Also, retreatment with corticosteroids after relapse was ineffective in the majority of relapsed patients. Overall, corticosteroid

treatment produced a prolonged remission in 13 patients (42%) of the total cohort of 31 patients for whom follow-up data was available. This is a higher success rate of corticosteroid therapy than previously reported in the systematic review of the literature that studied case reports with a variable follow-up (28%).[23] In case of treatment failure, combination therapy of rituximab and azathioprine or mycophenolate still achieves an approximate 75% remission rate and can be re-used even after relapse. [30]

High-dose corticosteroid treatment seems not only efficient but also safe for patients with gI-ILD. Although two patients suffered from opportunistic infections during corticosteroid treatment and none in the control group, these opportunistic infections occurred during maintenance therapy and not when corticosteroids were stopped within 12 months. Moreover, corticosteroid treatment was not associated with a detectable increase in respiratory tract infections and did not lead to a deterioration of airway disease within two years after corticosteroids were started.

Unfortunately, other corticosteroid related complications as well as potential prophylaxis for these complications (e.g., bisphosphonates for osteoporosis) were not specifically monitored in this study, which makes it difficult to interpret reported side effects and relate them to causality, dose and duration of corticosteroid therapy. The reported medical history suggested an increased rate of osteoporosis related complications in corticosteroid treated patients and osteoporosis related fractures seemed to be associated with maintenance therapy. Based on these results, osteoporosis should be actively monitored in gI-ILD patients that receive corticosteroid treatment and multiple risk factors for osteoporosis and we believe osteoporosis related fractures are a relevant contraindication for corticosteroids in the treatment of gI-ILD given the alternatives.

Serum sIL-2R, IgM and neopterin have been reported as potential biomarkers for gI-ILD.[13,32,33] sIL-2R and IgM have been shown to correlate with gI-ILD progression and response.[13,32] We found these biomarkers were outside the normal range at baseline in both groups. Moreover, the biomarkers were higher in the corticosteroid treatment group, showed clear correlations with parameters associated with gI-ILD severity, and were responsive to corticosteroid treatment.

Patients that received corticosteroid treatment reported more pain, fatigue, reduced physical functioning, hampered physical activity and dyspnea at baseline. However, only the fatigue, physical activity and dyspnea scores were significantly

lower than those reported for healthy individuals.[34,35,36] Corticosteroid treatment induced a large improvement of the dyspnea score, according to the CRQ, but not to other QoL domains.[35,36] In contrast, abatacept treatment also improved fatigue.[15] This suggests that dyspnea, fatigue and hampered physical activity are important health-status indicators in gl-ILD that should be clinically monitored.

Although STILPAD is the largest and first prospective international multisite study on gl-ILD in CVID, despite extensive analyses on potential confounders, the observational character, the mixed retrospective and prospective design and lack of standardized evaluation of the primary outcomes during baseline and follow-up are important limitations of this study that can introduce selection bias.

Thus, it is still possible that clinicians decided to give corticosteroid monotherapy and chose a tapering regimen based on unknown variables we could not correct for. Hence, this potential selection bias could reduce the robustness of our findings.

Still, there was no indication that patients who responded nor patients who achieved a prolonged remission to steroids had a less stringent diagnostic work-up or less severe disease compared to non-responders. In fact, patients who showed prolonged clinical response had more severe PFT and HRCT aberrancies, and more frequently had an expansion of $CD21^{low}$ B cells.

Moreover, since half of the patients included in the primary analysis had received their corticosteroid treatment partially or fully during the retrospective phase of the study, prospectively collected data, such as biomarker levels, QoL and infection rate were not recorded. Therefore, fewer patients were included in these analyses resulting in a limited power.

The lack of histologic confirmation in all patients might be considered a limitation, however, 90% of the patients that were included in the primary endpoint analysis had a probable gl-ILD diagnosis either based on histologic confirmation or a high gl-ILD probability score. Moreover, a subgroup analysis that included only patients with a probable gl-ILD diagnosis yielded similar results as the primary endpoint analysis.

In conclusion, high-dose corticosteroid treatment (≥0.03mg/kg) can improve gl-ILD in CVID patients. This effect was long-lasting and without major side effects in 42% of the patients, which provides an evidence-based rationale to

consider steroids as first-line therapy in CVID gl-ILD as suggested by the BLF/UKPIN [21] and which is of special relevance for areas in the world where other regimens might be less accessible. In addition, our data does not provide evidence for maintenance or repetitive high-dose steroid therapy. Thus, it appears to be strongly recommendable to switch to other immunosuppressive regimens after therapy failure, or when corticosteroid related side effects are anticipated or occur. Currently, we don't have markers that differentiate between patients who can be treated successfully with one course of corticosteroids and patients who will need other immunosuppressive regimens at first line.

sIL2R, Neopterin and IgM serum levels are suitable laboratory markers and should be added to the routine clinical evaluation of gl-ILD patients.

Future research urgently needs to perform the first controlled prospective interventional studies for gl-ILD investigating not only gl-ILD related outcome parameters but also QoL of the patients and biomarkers that can identify potential non-responders at baseline in order to provide more effective and safe alternative treatment modalities for all CVID patients with gl-ILD.

Acknowledgements
We would like to thank W. Vach and S. Weber for the initial biometric consultation, C. Winterhalter for her excellent support in data management, the many physicians and nurses supporting this international study and especially all patients and their families for their contribution. We also would like to thank the team of the CCI Biobank for their support. B.G. is funded by the Deutsche Forschungsgemeinschaft (GR1617/14-1/iPAD; SFB1160/2_B5; RESIST–EXC 2155–Project ID 390874280; the EU-H2020-MSCA-COFUND EURIdoc programme (No. 101034170) and the BMBF (GAIN 01GM1910A).

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SUPPLEMENTARY TABLES

Electronic Repository Table 1: *Overview of documented parameters during the trial*

Parameter	Retrospective (if available)	Baseline visit	Regular visit (every 6-12 months)	Additional visit[a]	Final visit (5 years after baseline)
Demographic characterstics		x			
Genetics					x (if available)
Medical history	x	x	x	x	x
infections	x (only major events)	x	x	x	x
Need of LTOT		x	x	x	x
Smoking status	x	x	x	x	x
Immunosupressive therapy	x	x	x	x	x
IgGRT	x	x	x	x	x
Concomitant medication		x	x	x	x
Physical examination		x	x	x	x
Ig levels	x (initial values)	x	x	x	x
immunophenotyping	x (latest values, prior to relevant IS)				
Biomarkers sIL2R, neopterin, IL6	x (sIL2R)	x	x	x	x
PFT (DLCOcSB, DLCOc/VA, TLC, FVC, FEV1, pO2 or O2 saturation at rest)	x (all available measurements)	x	x (every 12 mos.)	x	x
Exercise testing	x (all available measurements)	x	x (every 24 mos.)	x	
Chest CT scan (digital version)	all available scans, indication at discretion of investigator (recommended every 24 mos.)				
BAL analysis	x (all available measurements)				
lung histology	Centrally performed re-analysis of all available samples				
QoL CRQ-SAS, SGRQ		x	x	x	x
QoL SF-36		x	x (every 12 mos.)		x

[a] visits being scheduled because of pulmonary complaints, change of immunosuppression or 3-6 months after increase of immunosuppression

LTOT long term oxygen therapy, *IgGRT* IgG replacement therapy, *Ig* immunoglobulin, *sIL2R* soluble interleukin-2 receptor, *IL6* interleukin-6, *PFT* pulomonary function testing, *DLCOcSB* diffusion capacity of the lung single breath, *DLCOc/VA* diffusion capacity of the lung per alveolar volume, *TLC* total lung capacity, *FVC* functional lung capacity, *FEV1* forced expiratory volume in one second, *BAL* bronchoalveolar lavage, *QoL* quality of life, *CRQ-SAS* Chronic Respiratory Questionnaire Self-Administered Standardized, *SGRQ* St George's Respiratory Questionnaire , SF-36 36-Item Short Form Health Survey

Electronic Repository Table 2: *Overview of included patients*

ID	Genetic diagnosis	Start Mono-therapy	Stop Mono-therapy	Start Dose (mg/kg)	Taper regimen within first year of therapy	End of follow-up	Comment on end of follow-up	Primary Analysis	Follow-up	Secondary Analyses
P1	No	07/2007	01/2018	0.47	Maintenance	01/2018	End of study	Yes	Yes	Yes
P2	NA	05/2015	01/2018	0.90	Maintenance	01/2018	End of study	Yes	Yes	Yes
P3	No	07/2004	01/2006	0.68	Maintenance	01/2018	End of study	Yes	Yes	Yes
P4	No	02/2010	06/2010	0.4	Stop	01/2018	End of study	Yes	Yes	Yes
P5	No	12/2011	01/2018	0.31	Maintenance	07/2016	Start Rituximab	Yes	Yes	Yes
P6	No	12/2012	01/2018	0.57	Maintenance	01/2018	End of study	Yes	Yes	Yes
P7	No	01/2011	01/2018	0.37	Maintenance	01/2018	End of study	Yes	Yes	Yes
P8	No	10/2011	09/2015	0.90	Slow taper	02/2016	Start Sirolimus	Yes	Yes	Yes
P9	No	11/2014	08/2015	0.34	Stop	01/2018	End of study	Yes	Yes	Yes
P10	NA	05/2012	08/2012	1.09	Stop	11/2014	Start Azathioprine	Yes	Yes	Yes
P11	No	11/2014	10/2015	0.87	Maintenance, slow taper	07/2015	Start MMF	Yes	Yes	Yes
P12	No	05/2001	10/2005	1.23	Maintenance, slow taper	05/2008	Start MTX	Yes	Yes	Yes
P13	NA	05/2015	07/2015	0.62	Stop	01/2018	End of study	Yes	Yes	Yes
P14	No	09/2007	01/2018	0.41	Maintenance	12/2010	Start Rituximab	Yes	Yes	No
P15	No	07/2013	01/2018	0.49	Maintenance	01/2018	End of study	Yes	Yes	No
P16	NA	02/2013	05/2013	0.41	Stop	01/2018	End of study	Yes	Yes	No
P17	CTLA4	05/2013	07/2015	0.93	Maintenance	05/2015	Start Sirolimus	Yes	Yes	No
P18	No	01/2013	04/2013	0.41	Stop	07/2014	New course of high-dose CS	Yes	Yes	No
P19	No	05/2011	12/2012	0.70	NA	01/2018	End of study	Yes	No	Yes
P20	NA	04/2016	05/2016	0.67	Stop	01/2018	End of study	Yes	No	Yes
P21	No	01/2013	01/2018	0.58	Maintenance	06/2013	Start Rituximab	Yes	No	Yes
P22	No	02/2009	07/2009	0.86	Stop	01/2018	End of study	Yes	No	Yes

Electronic Repository Table 2: *Continued from previous page.*

ID	Genetic diagnosis	Start Mono-therapy	Stop Mono-therapy	Start Dose (mg/kg)	Taper regimen within first year of therapy	End of follow-up	Comment on end of follow-up	Primary Analysis	Follow-up	Secondary Analyses
P23	No	06/2014	12/2016	0.35	Stop, slow taper	10/2017	Start Abatacept	Yes	No	No
P24	No	02/2007	04/2007	0.42	Stop	01/2018	End of study	Yes	No	No
P25	No	11/2011	02/2012	0.88	NA	05/2012	Start Cyclosporine	Yes	No	Yes
P26	NFKB1	12/2013	04/2014	1.05	Stop	04/2015	Deceased	Yes	No	Yes
P27	NA	06/2013	11/2013	1.10	Stop	08/2014	Start Azathioprine	Yes	No	Yes
P28	NA	12/2015	01/2018	0.54	Maintenance, slow taper	03/2017	Start MMF	Yes	No	Yes
P29	No	12/2013	01/2018	0.44	NA	03/2014	Start Hydroxychloroquine	Yes	No	Yes
P30	NA	06/2011	03/2012	0.63	Stop	03/2012	Start Rituximab	Yes	No	Yes
P31	No	07/2016	01/2018	0.73	Maintenance	02/2017	Start MMF	Yes	No	Yes
P33	No	07/2014	07/2015	1.06	Stop	11/2015	Start Rituximab	Yes	No	Yes
P34	No	01/2003	12/2010	1.15	Maintenance	01/2018	End of study	Yes	No	Yes
P35	No	03/2009	01/2012	0.76	Stop, slow taper	01/2018	End of study	Yes	No	Yes
P36	NA	07/2011	09/2017	0.94	Maintenance, slow taper	01/2018	End of study	Yes	No	Yes
P37	NA	06/2012	NA	0.66	NA	01/2013	CS stop date unknown	Yes	No	No
P38	NA	08/2011	NA	1.07	Maintenance	05/2012	End of study	Yes	No	No
P39	No	06/2006	NA	0.47	NA	06/2007	CS stop date unknown	Yes	No	No
P40	No	01/2013	01/2018	0.45	Maintenance	01/2018	End of study	No	No	Yes
P41	NA	03/2016	07/2016	0.76	Stop	06/2016	MMF	No	No	Yes
P42	NFKB1	09/2016	03/2017	0.89	Stop	01/2018	End of study	No	No	Yes
P43	CTLA4	07/2008	07/2014	0.78	Maintenance	11/2009	Start Cyclosporin A	No	No	Yes
P44	NA	02/2001	01/2018	2.12	Maintenance	01/2018	End of study	No	No	Yes

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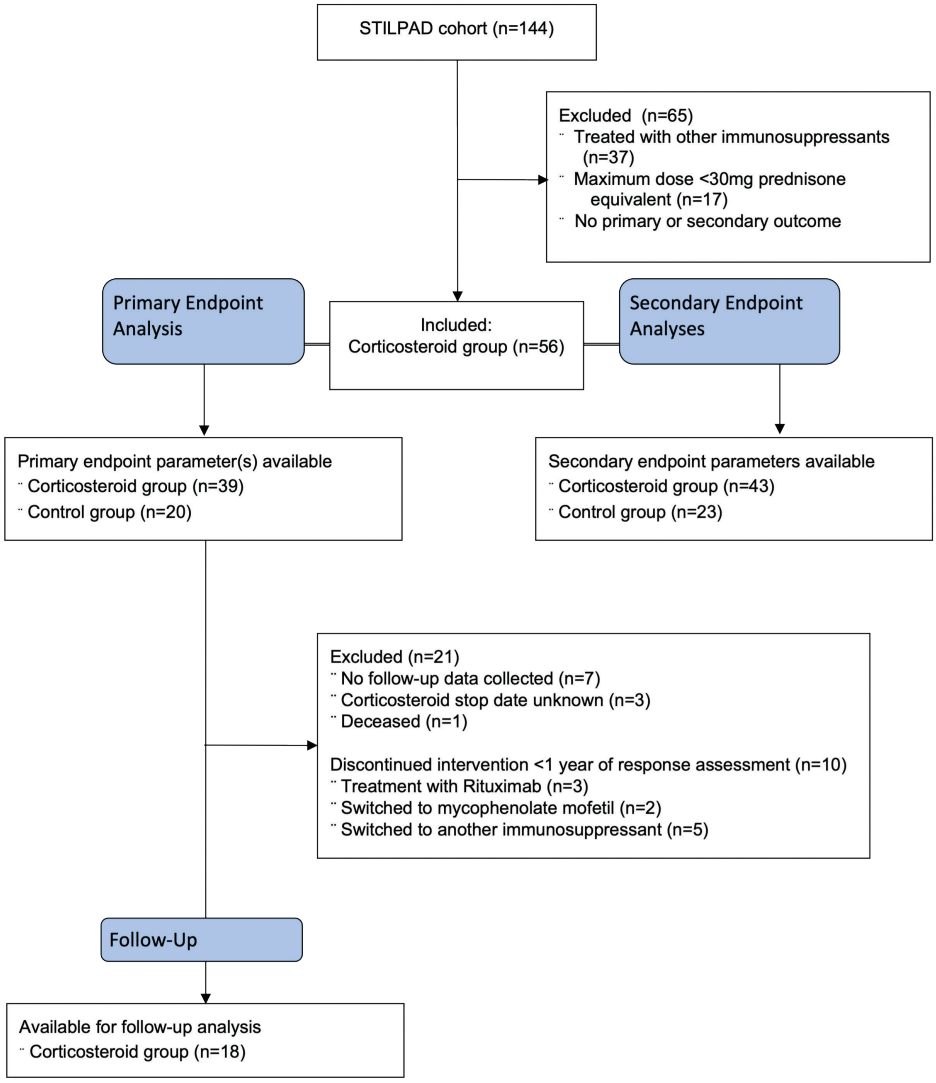
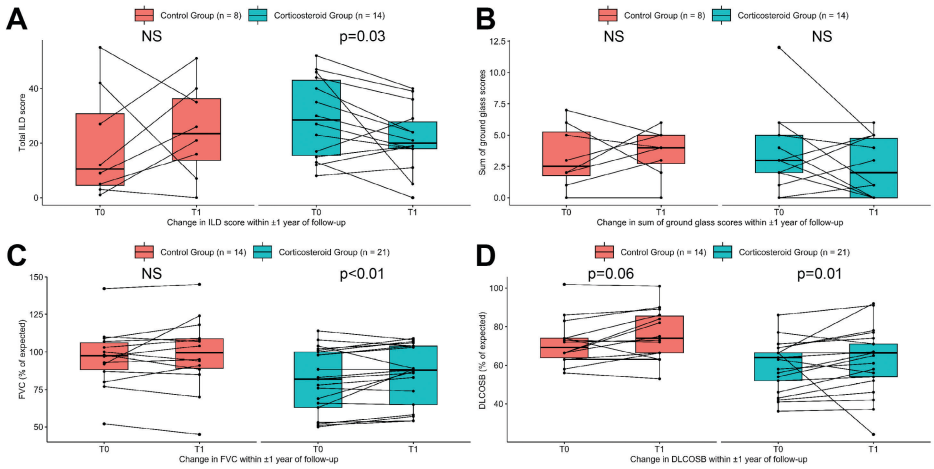
ID	Genetic diagnosis	Start Mono-therapy	Stop Mono-therapy	Start Dose (mg/kg)	Taper regimen within first year of therapy	End of follow-up	Comment on end of follow-up	Primary Analysis	Follow-up	Secondary Analyses
P45	NA	05/2008	12/2008	0.29	Stop	01/2018	End of study	No	No	Yes
P46	TACI	09/2013	01/2018	0.85	Maintenance	01/2018	End of study	No	No	Yes
P47	NA	07/2006	12/2012	0.52	Maintenance	12/2008	Start Azathioprine and Cyclyosporine A	No	No	Yes
P48	No	01/2016	02/2016	0.33	Stop	01/2018	End of study	No	No	Yes
P49	No	06/2010	08/2012	0.45	Maintenance	08/2010	Start Azathioprine	No	No	Yes
P50	TACI	03/2001	07/2003	0.63	Maintenance	07/2003	Start Azathioprine	Yes	No	No
P51	NA	12/2011	05/2012	0.41	Stop	01/2013	Start chemotherapy including Rituximab	No	No	Yes
P52	CTLA4	12/2014	04/2015	0.76	Stop	06/2015	Start MMF	No	No	Yes
P53	No	06/2012	02/2013	0.96	Stop	01/2018	End of study	No	No	Yes
P54	NA	01/2005	08/2006	0.54	NA	01/2018	End of study	No	No	Yes
P55	No	12/1995	07/1997	1.05	Stop, slow taper	04/2016	Start Hydroxychloroquine	No	No	Yes
P56	NA	06/2004	11/2010	0.93	Maintenance	01/2018	End of study	No	No	Yes
P57	NA	08/2004	03/2006	0.5	Stop, slow taper	01/2018	End of study	No	No	Yes

SUPPLEMENTARY FIGURES

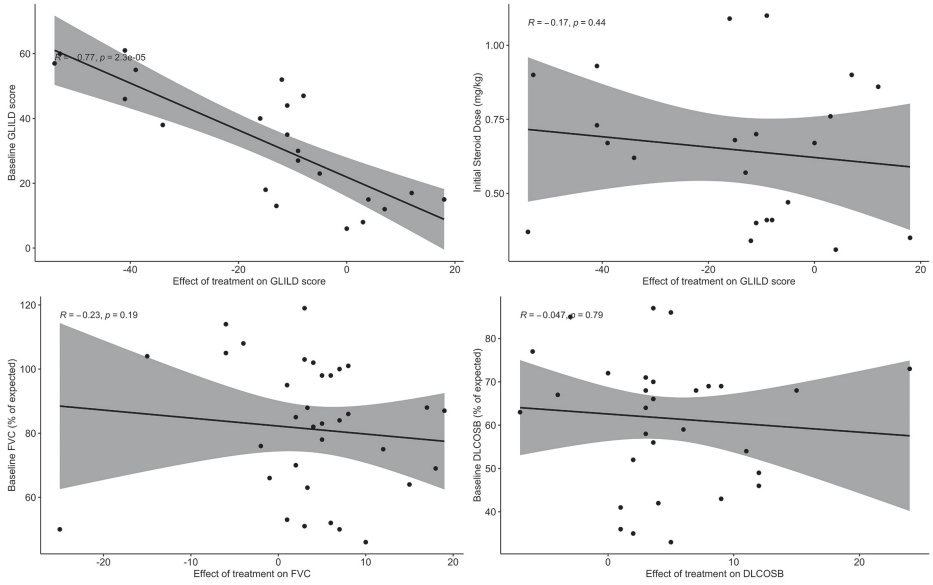
Electronic Repository Figure 1: Patients that received high-dose corticosteroids were selected from the STILPAD cohort. *Patients that were treated with ≥0.03mg/kg and ≥30mg of prednisone equivalent were selected from the STILPAD cohort and included in the primary endpoint analysis if HRCT and/or PFT data was collected at the start of therapy and within 24 months after the start of therapy and were included in secondary analyses if the appropriate data was available. Patients that responded to corticosteroids were then considered for follow-up analysis. Patients were excluded for follow-up, when follow-up data was insufficient or when patients were switched to other immunosuppressive regimens before follow-up assessment was performed.*



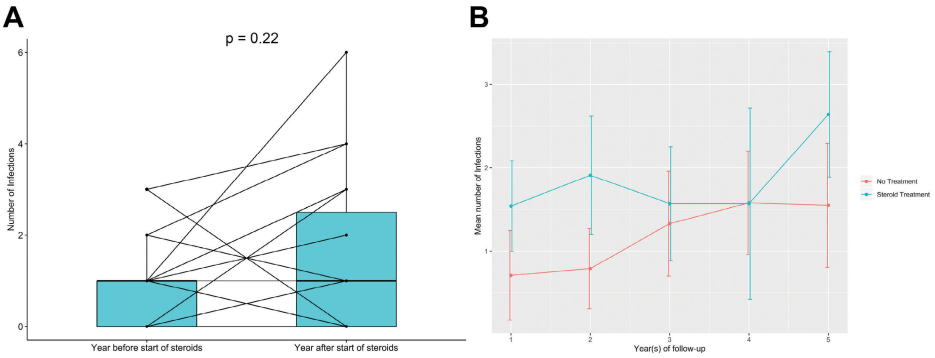
Electronic Repository Figure 2: Corticosteroid treatment leads to significant improvement of ILD related HRCT scores and PFT parameters. A-B *The ILD score and Ground Glass sum score measured at baseline and within one year of follow-up for the control group and corticosteroid treated patients. Total ILD was calculated by adding up the Nodules, Ground Glass and Reticulation sub scores of all lung fields. Patients treated with corticosteroids show a significant reduction of the ILD score.* **C-D** *Forced vital capacity (FVC) and diffusion capacity of the lung for carbon monoxide in a single breath ($DLCO_{SB}$) at baseline and within one year of follow-up. Patients treated with corticosteroids show a significant improvement of both FVC and $DLCO_{SB}$.*



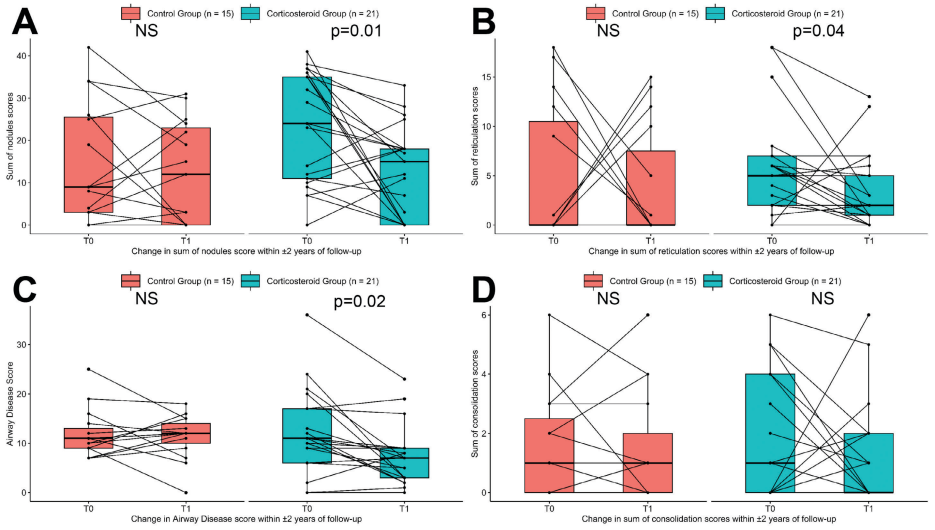
Electronic Repository Figure 3: The baseline ILD score correlates with the total effect of corticosteroid treatment. *High baseline ILD scores correlate with higher reductions of the ILD score, while corticosteroid dose did not correlate with ILD score reduction. This effect was not seen for the PFT outcomes.*



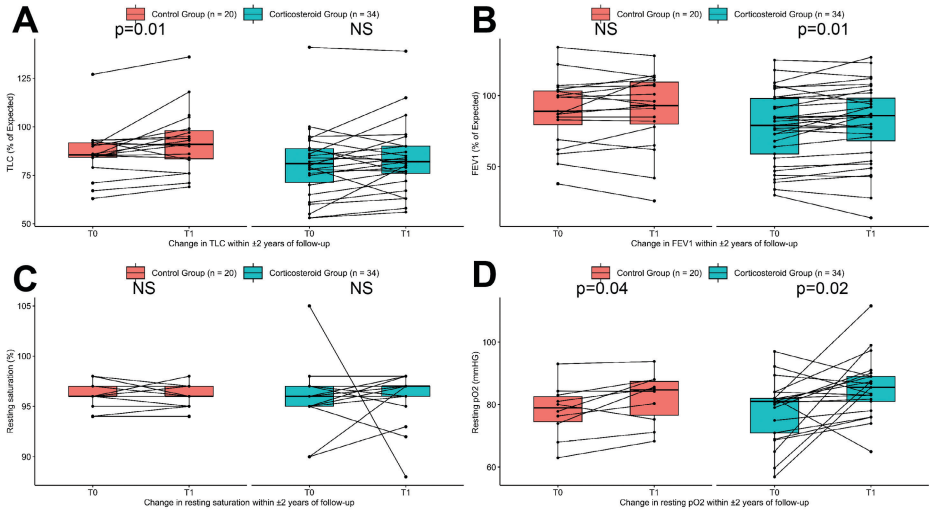
Electronic Repository Figure 4: Corticosteroid treatment does not increase the number of infections. ***A*** *The number of infections in the year before and the year after the start of corticosteroids were not significantly different (Wilcoxon-signed rank test). The mean number of infections over time did not differ between the corticosteroid group and the control group (Mann-Whitney U test).*



Electronic Repository Figure 5: Corticosteroid treatment leads to significant improvement of nodules, reticulation and airway disease. A-D *The nodules sum score and reticulation sum score, airway disease score, and consolidation sum score measured at baseline and within two years of follow-up for the control group and corticosteroid treated patients. Patients treated with corticosteroids show a significant reduction of nodules, reticulation and airway disease.*



Electronic Repository Figure 6: Corticosteroid treatment leads to significant improvement of FEV1 and pO_2. A–D *Total lung capacity (TLC) and forced expiratory volume in 1 second (FEV1), resting saturation, and resting pO_2 at baseline and within two years of follow-up measured in the control and corticosteroid group. Patients treated with corticosteroids show a significant improvement of the FEV1 and pO_2, while the control group only showed significant improvement of the TLC and pO_2.*



CHAPTER 11

A minimal parameter set facilitating early decision-making in the diagnosis of hemophagocytic lymphohistiocytosis

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ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening immune dysregulation syndrome characterized by uncontrolled immune cell activation. Timely diagnosis is important, since early treatment can improve survival rates. However, completing all assessments to reach >_5 positive criteria out of the 8 HLH-2004 criteria can be time-consuming and may delay timely initiation of treatment. Hence, we applied a data driven approach to identify a minimal parameter-set for early decision making.

We retrospectively evaluated 165 patients from five Dutch tertiary hospitals suspected of having the HLH syndrome. Clustering analysis and multi-receiver operator characteristics were used to identify the parameters most distinctive to predict the HLH syndrome.

Sixteen pHLH (median age 0.5 years) and 70 sHLH patients (median age 8.7 years) were identified using the HLH-2004 criteria. All HLH cases had either increased ferritin, cytopenia in ≥2 lineages or splenomegaly, which distinguished them from non-HLH cases with a negative predictive value of 100%. A minimal parameter set consisting of 2 major criteria (phagocytosis and splenomegaly) and 3 minor criteria (cytopenia, increased ferritin and increased triglycerides/low fibrinogen) predicted HLH with 95% (88 – 99) sensitivity and 94% (86 – 98) specificity. This finding was replicated in an independent retrospective validation cohort of 109 US patients (n=109).

By dividing a subset of the HLH-2004 criteria into major and minor criteria, this strategy uses the evaluation of less than 5 criteria to quickly identify patients with the HLH syndrome. When confirmed in a prospective setting, this approach could be of value for timely diagnosis and treatment of the HLH syndrome.

Key words
HLH; Hemophagocytic lymphohistiocytosis; Diagnostic criteria; Clustering analysis

ABBREVIATIONS

HLH = Hemophagocytic Lymphohistiocytosis
sHLH = secondary HLH
pHLH = primary (familial) HLH
sIL-2R = soluble interleukin 2 receptor
PLS-DA= partial least squares differential analysis
AUC = area under the curve
PCA = Principal Component Analysis
multiROC = multi Receiver Operator Characteristics

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening immune dysregulation syndrome characterized by uncontrolled immune cell activation.[1,2] Activated lymphocytes and macrophages produce a cytokine storm that induces hemophagocytosis and tissue phagocytosis by over-activated macrophages.[1,3,4] HLH eventually results in multiple organ failure that culminates in mortality in up to 50% of affected children and adults.[1,3,5,6]

The two sub-types of HLH include primary HLH (pHLH) and secondary HLH (sHLH). pHLH is a group of diseases caused by germ line mutations in genes involved in vital immunologic pathways.[7,8] Defective T- and NK lymphocyte function presumptively causes a failure in the termination of the immune response as well as persistent antigenemia that stimulates the immune system and induces the cytokine storm.[9,11,12] Secondary HLH can be induced by infection (42%), cancer (40%) or auto-immune disease (11%).[1,4,11-15]

The diagnosis of HLH syndrome forms a diagnostic challenge since the symptoms can be similar to other, more common diseases.[16,17] HLH syndrome is currently diagnosed according to the presence of at least 5/8 of the HLH-2004 criteria: fever, splenomegaly, increased ferritin, high soluble IL-2R, cytopenia in at least two cell lineages, tissue phagocytosis, low NK lymphocyte mediated lysis of target cells and either high triglycerides or low fibrinogen.[18] Since the underlying triggers for HLH syndrome vary, it is assumed to be a heterogeneous disease and there is debate whether this has impact for the diagnostic process.[19,20] For example, there is evidence that patients with HLH syndrome due to malignancy present with different HLH related parameters when compared to HLH patients with other underlying diseases.[20-22] Lastly, the diagnostic properties of the NK lysis assays and the sIL-2R assays remain uncertain for the diagnosis of the HLH syndrome. These laboratory tests can be time-consuming and are restricted to specialized centers, which may delay definitive diagnosis and treatment.[18-21]

Early recognition of HLH syndrome is imperative as timely administration of immunosuppressant drugs prevents aggravation of immune dysregulation, as was shown in a previous retrospective analysis showing a correlation between early etoposide administration and survival.[24]

Since the current HLH criteria involve a subset of laborious tests, one has to ask whether HLH-treatment should be withheld until 5/8 positive criteria are found, or if a minimal parameter set could also be decisive for HLH diagnosis.

Hence, the objective of this study was to identify a minimal parameter set required to predict HLH syndrome that could serve as a tool for early therapeutic decision-making and avoid the need to wait for specialized sIL-2R and NK function testing. For this, data driven statistical methods were used in a Dutch retrospective discovery cohort of HLH suspected children and adults. The minimal parameter set was subsequently confirmed in an American retrospective replication cohort of HLH suspected adults.

METHODS

Clinical record review and cohort acquisition

After approval of the medical ethical committee, (METC nr. 17/111), we retrospectively evaluated the clinical records of 264 patients suspected of HLH from 5 academic medical centers in the Netherlands in whom NK/lymphocyte function tests or sIL-2R assays were performed between 2006 and 2016. After reviewing the patient records, the records of 99 patients were excluded since too many HLH-2004 criteria were missing to exclude HLH syndrome. The records of 165 patients were selected for further review. Fulfilment of ≥5 of the 8 HLH-2004 criteria and/or confirmed germline mutations associated with pHLH were used to establish HLH diagnosis. The results of all 8 HLH-2004 criteria assessments were collected from the electronic records together with patient characteristics such as age, gender and final diagnosis.

NK/lymphocyte function and sIL-2R analyses

NK lymphocyte functional testing was performed in a central diagnostic reference center (Laboratory of Translational Immunology, UMC Utrecht) using CD107a degranulation and NK- cell lysis assays. For both assays, PBMC's were isolated and cell subsets were counted. For the CD107a assay, PBMC's were incubated with K562 tumor cells and CD8+ CD107a+ cells were counted using flow cytometry. For the NK lysis assay, two effector (NK lymphocytes) vs target (K562 tumor cells) ratios (1:1 and 2:1) were used and NK lysis was analyzed using Celltrace violet© and flow cytometry. Moreover, NK lymphocyte function panels from healthy siblings that weren't investigated for HLH were included for the analysis of the sensitivity and specificity of the NK lymphocyte function assays.

Soluble interleukin 2 receptor (sIL-2R) was measured in individual hospitals using the Diaclone© (upper limit of normal: 2500 pg/mL) or Immulite© (upper limit of normal: 7500 pg/mL) enzyme-linked immunosorbent assays (ELISA) according

to the manufacturer's instructions. We normalized the data by calculating a fold-change relative to the upper limit of normal of the used test kit.

Validation cohort

The validation cohort consisted of 109 sHLH patients and 38 non-HLH patients from the Hematology Division of Johns Hopkins Hospital in Baltimore, Maryland. These patients were retrospectively acquired from billing and lab results and classified according to the HLH-2004 criteria between January 1, 2009 and August 1, 2018 as previously published.[25] Diagnostic criteria were recorded and peak values for ferritin and triglycerides and fibrinogen nadir values were also noted. HLH was defined by the HLH-2004 criteria. Data collection was approved by the Johns Hopkins Institutional Review Board. Data on the patients was de-identified by the treating physician before data analysis.

Statistics

Statistics were performed in R studio 1.1.456, which was used to compare proportions of positive parameters between the different groups of HLH patients and produce a heatmap of these parameters. Then the "mixomics" package was used for a partial least squares discriminant analysis (PLS-DA) to find characterizing parameters in a supervised method. Furthermore, the "psych" and "GPArotations" packages were used to perform a Principal Component Analysis (PCA), after imputation with the "amelia" package, to find characterizing parameters in an unsupervised method.

Moreover, multi–receiver operator characteristics (multiROC) curves were calculated by using generalized linear modelling with pROC and R's inbuilt statistics packages to compare the diagnostic models. Lastly, cut off values for NK/lymphocyte function were defined by calculating the Δ and the fold change of the 1:1 vs 2:1 dilution. For this, R's "Optimal.Cutpoints" and "epiR" packages were used to calculate sensitivity and specificity of the proposed new algorithms.

RESULTS

Prevalence of the 8 HLH-2004 criteria in HLH subgroups

One hundred sixty-five HLH-suspected patients were included in the primary cohort of which 86 met the HLH-2004 criteria. The median age of the pHLH group was 0.5 (0 – 50.7) years, 8.7 (0 – 83) years in the sHLH group and 9.7 (0 – 84) years in the non-HLH group. The most common primary diagnosis in the non-

HLH group was either auto-immune disease (20.8%) or immunodeficiency (20.8%), whereas infection (27%) and malignancy (24.3%) were the most common causes of sHLH (Table 1). There also was significant variation in positive criteria between HLH subgroups especially for the number of cytopenias and the NK-lysis assay, which were more often normal in some of these subgroups. Moreover, significant variation existed within the HLH subgroups, denoted by the large standard deviations that were found. To identify common denominators of HLH between these groups we first tried a steered approach, followed by several clustering methods.

Table 1: Summary statistics on included patients. *Non-HLH patients were defined as patients who did not meet the HLH-2004 criteria. The type of underlying disease was defined as the primary diagnosis that the patient was suffering from (e.g. a patient suffering from SLE and infection was scored as Auto-Immune; AI) The largest variation in symptom positivity was seen in the presence of cytopenias and an aberrant NK lysis assay. Moreover splenomegaly, cytopenia and elevated ferritin were frequently encountered in HLH patients.*

	Non-HLH (n=79)	**pHLH (n=16)**	**AI (n=11)**	**Infection (n=20)**	**PID (n= 7)**	**Cancer (n=18)**	**Unknown (n=14)**
Median Age (range)	9 (0 – 84)	1 (0 – 51)	16 (1 – 40)	7 (0 – 47)	4 (0 – 64)	15 (1 – 83)	2 (0 – 58)
Gender (male)	64.5%	50%	63.6%	50%	100%	61.1%	71.4%
Fever > 38.5 °C (NA)	62.0% (0)	93.8% (0)	100% (0)	95.0% (0)	71.4% (0)	94.4% (0)	76.9% (1)
Splenomegaly (NA)	20.3% (0)	81.3% (0)	72.7% (0)	80.0% (0)	85.7% (0)	77.8% (0)	78.6% (0)
Proven HLH on biopsy (NA)	5.3% (41)	64.3% (2)	66.7% (2)	58.8% (0)	100% (0)	60.0% (3)	71.4% (0)
Cytopenias ≥ 2 (NA)	20.3% (0)	93.8% (0)	45.5% (0)	68.4% (1)	100% (0)	83.3% (0)	64.3% (0)
Ferritin ≥ 500 (NA)	67.7% (14)	100% (0)	90.9% (0)	100% (0)	50% (1)	100% (0)	100% (0)
Ferritin ≥ 1000 (NA)	43.1% (14)	81.3% (0)	81.8% (0)	80% (0)	50% (1)	83.3% (0)	85.7% (0)
- Mean ferritin levels (SD)	5020 (13662)	3379 (2597)	12106 (10476)	28689 (101621)	5576 (9017)	7510 (7825)	11139 (21214)
↑ Triglycerides/ ↓ Fibrinogen (NA)	31.8% (35)	86.7% (2)	63.6% (0)	68.4% (1)	66.7% (1)	64.7% (1)	54.5% (3)
- Mean triglyceride levels (SD)	2.47 (1.90)	4.65 (2.11)	4.05 (2.23)	4.14 (1.62)	2.93 (3.03)	4.28 (2.53)	3.70 (1.90)
- Mean fibrinogen levels (SD)	3.97 (2.54)	2.81 (3.46)	2.51 (1.58)	2.63 (1.61)	1.90 (1.45)	3.39 (1.60)	5.25 (5.98)
↑ sIL-2R (NA)	50.0% (21)	100% (3)	66.7% (2)	87.5% (4)	83.3% (1)	100% (3)	83.3% (2)
- Mean X upper reference value (SD)	4.9 (7)	24.0 (22)	6.2 (5)	13.7 (11)	23.0 (34)	15.0 (13)	18.5 (22)
Aberrant NK-lysis assay (NA)	19.4% (48)	75% (12)	37.5% (3)	0% (9)	40% (2)	60% (13)	71.4% (6)
Median # of positive criteria (range)	2 (0-4)	7 (3-7)	5 (5-7)	6 (5-7)	5 (5-7)	6 (5-7)	5 (5-7)
CNS involvement (NA)	0% (49)	60% (1)	25% (3)	45.5% (9)	0% (2)	50% (6)	41.7% (1)

Our first approach was to evaluate parameters that are readily available in the clinical setting: fever, elevated ferritin, cytopenias in ≥2 cell lines and splenomegaly. These criteria might guide decision making towards additional HLH diagnostics. Hence, their performance as indicator of HLH was analyzed (Supplementary, table 1) and optimum cut-off values for ferritin were defined to yield 100% sensitivity (1000 µg/L). The presence of either splenomegaly, severe cytopenia in >2 lineages (according to the HLH-2004 criteria), or increased ferritin (>1000 ug/L) yielded 100% sensitivity and 65% specificity with a negative predictive value of 100% for HLH in the discovery cohort.

Hierarchical clustering

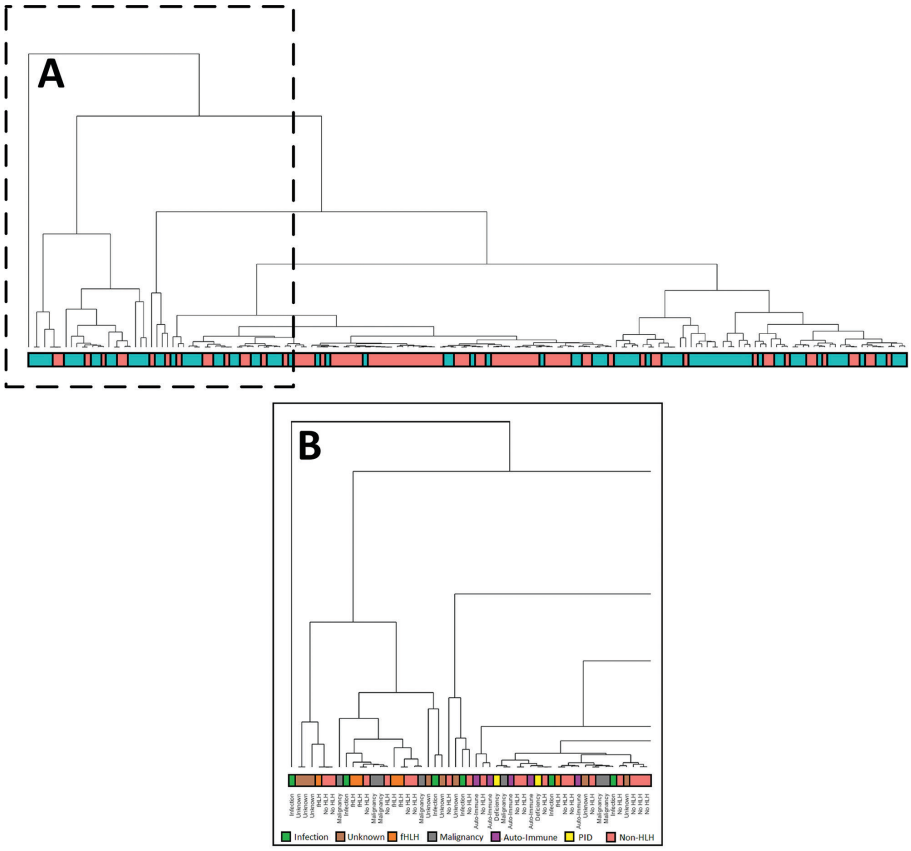
Our second approach was to use clustering analysis to find parameters that could distinguish between HLH and non-HLH patients and parameters that could distinguish between the different types of HLH. First, hierarchical clustering was used to analyze whether the 8 HLH-2004 criteria, CNS symptoms, NK lymphocyte numbers and bilirubin levels formed diagnostic clusters and whether these clusters could separate pHLH from sHLH patients and the separate subgroups within the sHLH spectrum. We found that even though no specific diagnostic clusters were formed, hierarchical clustering was moderately capable of separating the non-HLH patients from the HLH patients (Figure 1A). However, hierarchical clustering could not separate the pHLH from sHLH patients (Figure 1B), nor the separate subgroups within the sHLH spectrum. This suggests that there are no specific parameters that can distinguish the separate forms of HLH within the HLH-2004 criteria.

Supervised clustering with dimension reduction

Secondly, PLS-DA was used to maximize the chance of finding discriminating clusters of criteria that could define the sHLH subgroups and distinguish between sHLH and pHLH, but none could be found (Supplementary, figure 1). The criteria that could identify HLH patients in general, overlapped excessively for the pHLH and sHLH patients and also for the subgroups of sHLH. Hence, the HLH patients were pooled for further analysis.

The results of the pooled PLS-DA are shown in Figure 2A & 2B. The presence of splenomegaly, together with cytopenias, proven tissue hemophagocytosis, fever, increased sIL-2R and elevated triglycerides could distinguish HLH patients from non-HLH patients effectively with an area under the curve (AUC) of 0.93. Moreover, splenomegaly, biopsy proven hemophagocytosis and cytopenias are the most distinguishing parameters in this analysis.

Figure 1: Hierarchical clustering dendogram that could moderately separate non-HLH patients (red) from HLH patients (blue). *This clustering strategy could not distinguish pHLH from sHLH patients (A) nor the subtypes of sHLH (B), based on these parameters.*



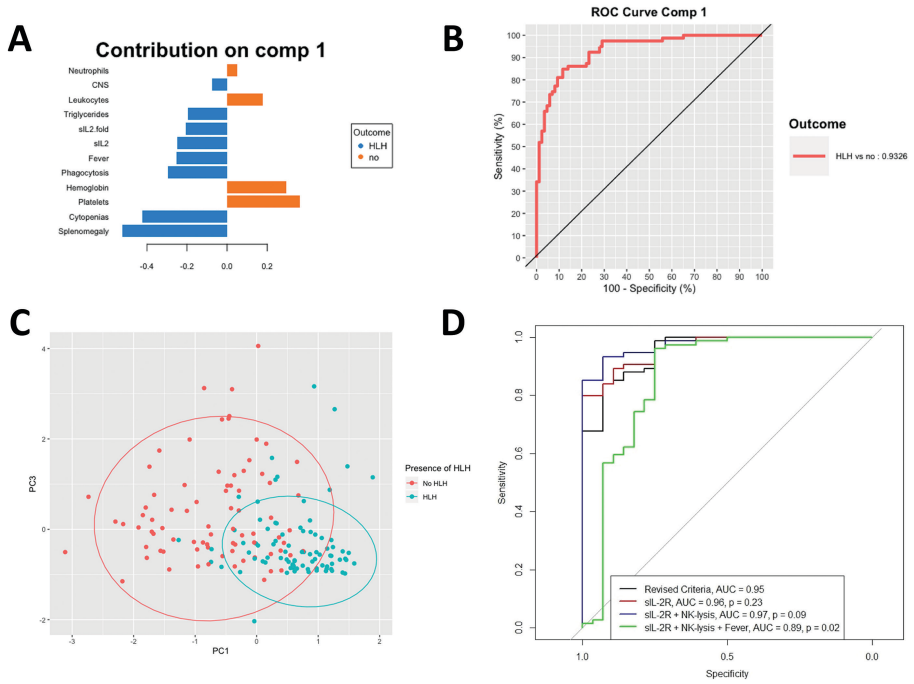
Unsupervised clustering with dimension reduction

Finally, a PCA analysis with oblique rotation was performed to see if an unsupervised approach would yield similar criteria. The Kaiser-Meyer-Olkin (KMO) measure showed that there were too few samples to explain all variables, which led to the exclusion of NK lysis 2:1, NK fold-change and age, since these had the poorest common variance. This resulted in an overall KMO=0.64 with no single value below 0.5. Bartlett's test of sphericity resulted in chi-squared (45) = 239, p<0,0001. A scree plot showed that four factors were able to explain most variance within the dataset, of which the first three explained 81% of the variance (Supplementary figure 2).

Cluster analysis showed that either PC1 or PC2 combined with PC3 could distinguish between the non-HLH and HLH patients (Figure 2C). The variance of PC1 was

mostly due to neutrophils (0.41), leukocytes (0.40) and platelets (0.26). For PC2 this was ferritin (0.50), NK lysis (0.49) and sIL-2R (0.26) and for PC3 tissue phagocytosis (0.46) and splenomegaly (0.19).

Figure 2: List of HLH defining symptoms that separate HLH patients from non-HLH patients *in a PLS-DA (A), with an AUC of 0.93 (B). PCA showed that either PC1 (cytopenias, 32%) or PC2 (Ferritin/NK-Lysis/sIL-2R, 25%) with PC3 (Splenomegaly/Proven biopsy of HLH, 21%) could separate HLH from non-HLH patients (C). Multi-ROC analysis wielded a combination of Splenomegaly, proven biopsy of HLH, Cytopenias, Elevated ferritin and ↑Triglycerides/↓Fibrinogen as minimal parameter set with an AUC of 0.95 which did not significantly improve with the addition of NK-lysis or sIL-2R (D).*



Simulating possible sets of minimal parameter sets with multiROC

Since tissue hemophagocytosis, splenomegaly and cytopenias were the most defining parameters for HLH in both the PLS-DA and the PCA, these criteria were used as initial parameter set. We then simulated the minimal parameter set needed for HLH diagnosis, by iteratively adding the criterion that caused the largest increase in AUC (Supplementary, figure 3). This ultimately led to the discovery of a combination of biopsy proven hemophagocytosis, splenomegaly, cytopenias in ≥2 lineages, ferritin ≥1000 and ↑triglycerides/↓fibrinogen with an AUC of 0.95. Further addition of the other criteria (fever, sIL-2R and aberrant NK/lymphocyte function assay) did not improve the algorithm (Figure 2D).

Furthermore, since splenomegaly and biopsy proven hemophagocytosis clustered together in the PCA and were also among the top discriminative parameters in the PLS-DA, these criteria were analyzed as major criteria. The remaining three criteria were analyzed as minor criteria and compared to the golden standard, the HLH-2004 criteria, as presented in table 2. HLH was deemed most likely when a patient had at least 2 major (48% sensitivity, 100% specificity), 1 major and 2 minor (79% sensitivity, 95% specificity) or 3 minor positive criteria (49% sensitivity, 97% specificity), with a combined sensitivity of 94% and specificity of 95%.

Table 2: Analysis of the sensitivity and specificity of the minimal parameter set that can predict HLH. *We used splenomegaly and tissue phagocytosis as major criteria and ferritin, cytopenia and triglycerides/fibrinogen as minor criteria. These were replicated in another retrospective cohort which produced similar results.*

Test	Discovery Cohort Sensitivity (CI)	Discovery Cohort Specificity (CI)	Replication Cohort Sensitivity (CI)	Replication Cohort Specificity (CI)
2 major +	0.48 (0.37 – 0.59)	1.0 (0.91 – 1.0)	0.44 (0.25 – 0.54)	1.0 (0.91 – 1.0)
1 major & 2 minor +	0.79 (0.69 – 0.87)	0.95 (0.88 – 0.99)	0.87 (0.79 – 0.93)	0.97 (0.86 – 1.0)
3 minor +	0.49 (0.38 – 0.60)	0.97 (0.91 – 1.00)	0.76 (0.67 – 0.84)	0.97 (0.86 – 1.0)
Minimal parameter set	0.94 (0.86 – 0.98)	0.95 (0.88 – 0.99)	0.98 (0.94 – 1.0)	0.95 (0.82 – 0.99)

Analysis of the minimal parameter set in the replication cohort

The replication cohort consisted of 109 sHLH patients with a median age of 58 (19 - 77) and 38 non-HLH patients with a median age of 54 (19 – 81). The most common primary diagnosis in the non-HLH group was autoimmune (47%), whereas malignancy (39.4%) and infection (36.7%) were the most common causes of sHLH in this cohort (Supplementary, table 2). Moreover, 9.8% of patients in this cohort died while waiting for the NK-lysis or the sIL2-R assay results, and concurrently met the HLH-2004 criteria after their death.

The presence of either splenomegaly, ≥2 cytopenias or ferritin ≥1000 µg/L, wielded 100% sensitivity and 16% specificity. The minimal parameter set, containing splenomegaly and tissue hemophagocytosis as major criteria and ≥2 cytopenias, ferritin ≥1000 µg/L, and increased triglycerides/decreased fibrinogen as minor criteria, could distinguish sHLH patients from non-HLH patients with 97% sensitivity and 95% specificity which confirmed that this was a sensitive and specific minimal parameter set that could identify the patients with HLH.

The role of the NK/lymphocyte function and sIL-2R assays

Even though the NK/lymphocyte function and sIL-2R assays are not included in the minimal parameter set, they are still part of the HLH-2004 diagnostic criteria. Hence, we measured their performance in this cohort as decisive fifth criterion in borderline positive cases. There were 26 cases in which HLH was diagnosed based on the minimum of 5 positive criteria. Since sensitivity of the NK/lymphocyte function assay was low (Table 3), it could only be used as fifth positive criterion in 6/26 cases. Hence, we calculated a cut-off for the dilution series with maximum sensitivity at a specificity of at least 90%, to improve the diagnostic properties of the NK-lysis assay that is currently used, without impairing its robustness. This wielded a cut off fold-change of 1.17 with an AUC of 0.602, which significantly improved the diagnostic properties the NK/lymphocyte function assay (Table 3).

sIL-2R has previously been suggested as a sensitive HLH marker and was important in our cohort since it was needed in 20/26 cases to get to 5 positive criteria.[26] To confirm previous research, the performance of sIL-2R as sole indicator of HLH was measured in our cohort. Cut offs were calculated with a minimum sensitivity of 90% and with a minimum specificity of 90%, which were 2.63 and 11.8 respectively with an AUC of 0.806 (Table 2). These results implicate that although NK-lysis and sIL-2R are not needed for initial treatment initiation, they can be used to acquire the five positive criteria needed for unambiguous diagnosis.

Table 3: *Analysis of the performance of the current NK lymphocyte function tests vs addition of the dilution series as sole predictor for HLH (* p<0,01) and sensitivity and specificity cut offs for sIL-2R as HLH predictor.*

Test	Sensitivity (95% CI)	Specificity (95% CI)
Lysis <5% and CD107a <7%	0.27 (0.07- 0.31) *	0.92 (0.83 - 0.97)
Foldchange <1.17	0.29 (0.16 - 0.45)	0.94 (0.84 – 0.98)
Combined Criteria	0.55 (0.39 – 0.70) *	0.87 (0.76 – 0.94)
sIL-2 > 2.63	0.93 (0.84 – 0.98)	0.40 (0.27-0.53)
sIL-2 > 11.8	0.42 (0.31 -0.55)	0.91 (0.81 – 0.97)

DISCUSSION

Early diagnosis of the HLH syndrome is indispensable for timely administration of therapy to prevent aggravation of immune dysregulation, clinical deterioration, and significant morbidity and mortality. In this retrospective cohort analysis, we evaluated the diagnostic value of the individual HLH-2004 criteria using novel statistical methods including unsupervised hierarchical cluster analysis and principal component analysis. We defined a minimal parameter set enabling the exclusion of HLH syndrome and a parameter set consisting of major and minor criteria for positive identification of HLH syndrome. Together, these parameter sets can facilitate early therapeutic decision-making for HLH.

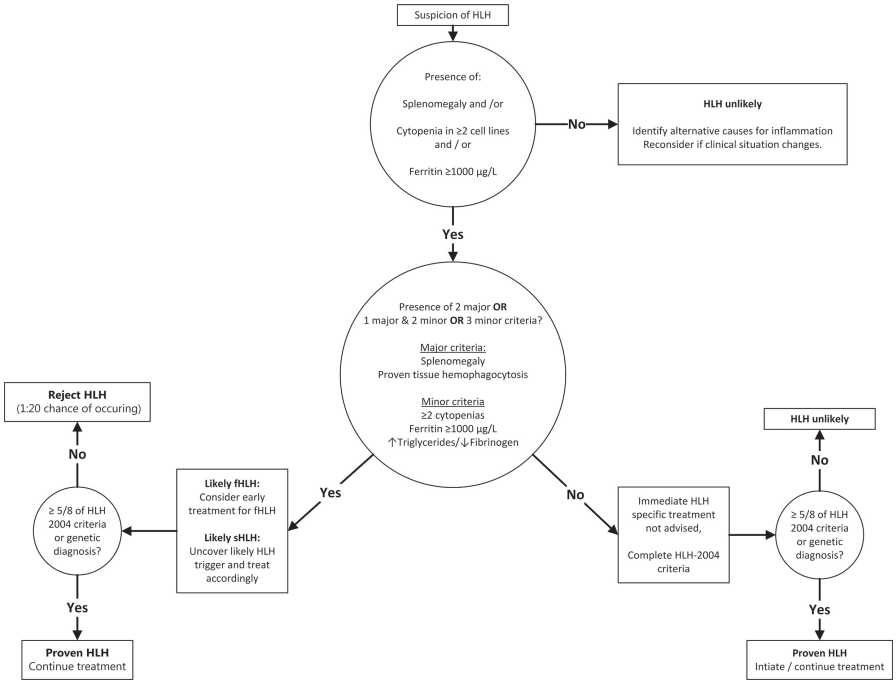
In a rigid diagnostic approach, a diagnosis of HLH is likely when 5 / 8 of the HLH-2004 criteria have been met. This may delay timely diagnosis, because several of these criteria require time consuming assays, which are not always widely available at most medical centers. Delayed diagnosis is especially threatening for severely ill patients. This was reflected by the fact that 9.8% of patients in the replication cohort died while waiting for one of these results, and subsequently met the HLH-2004 criteria after test results came available. Vice versa, early initiation of etoposide was shown to increase survival in a retrospective study in 162 sHLH patients. Thus, any effort to facilitate an early diagnosis is important to reduce the high mortality of HLH.[24]

Failure to meet the ferritin, cytopenia, and splenomegaly criteria excluded HLH in our cohort. At the same time, we found that this minimal parameter set consisting of 2 major criteria (hemophagocytosis and splenomegaly) 3 minor criteria (cytopenia, increased ferritin and increased triglycerides/low fibrinogen), which can all be available within 24 hours, predicted HLH with 95% (88 – 99) sensitivity and 94% (86 – 98) specificity.

As with the HScore, which is used to facilitate early identification of HLH in suspected cases,[27,28] the number of criteria needed to predict HLH occurrence was reduced. Fever, the NK lysis assay, and sIL-2R levels were excluded from our model as initial diagnostics, since they had less favorable diagnostic properties in our cohort. An advantage of our minimal parameter set is that we used the distinction of major and minor criteria and calculated the diagnostic properties of different sets of criteria, exactly predicting the combined strength of these sets.[26] More recently, other tools have been identified such as the MAS classification

criteria, the MS score and the ESR/ferritin ratio. These have however only been confirmed in patients with autoimmune disease related HLH and hence their performance in the entire spectrum of HLH syndrome is unknown. Since the minimal parameter set had a false positive rate of 1:20, we still suggest to confirm HLH in all suspected HLH patients with the full set of HLH-2004 criteria, combined with genetic confirmation and evaluation of secondary triggers where indicated, to prevent longtime exposure to immunosuppressants in non-HLH patients (Figure 3).

Figure 3: *Decision tree as minimal parameter set for diagnosing HLH and initiation of treatment.*



The results further showed that, in contrast to what has been shown in previous cohorts, individual criteria on their own lacked either specificity or sensitivity.[17,26,29,30] Patients with abnormal ferritin, sIL-2R and NK-lysis values formed one cluster, suggesting that these parameters are all indicators of severe systemic inflammation or critical illness. This is in line with other studies showing that NK lymphocyte mediated target cell lysis, cytokine production and CD107a surface receptor expression were lower in ICU patients compared to healthy controls.[31-33] The exclusion of the sIL-2R and NK function tests in the initial assessment of HLH syndrome suspected patients could help provide timely HLH diagnosis and improve diagnostic uncertainty.

Data on the diagnostic value of ferritin as sole marker for HLH syndrome remain inconclusive.[34,35] We now show that it can indicate patients for whom HLH diagnostics would be appropriate when combined with the criterion of splenomegaly and / or cytopenias.[17,36] We thus advocate the addition of ferritin measurement to the routine work-up of patients with systemic inflammation.[34,37-39]

Moreover, both classical statistical methods and clustering analysis could not distinguish pHLH from sHLH patients in patients suspected of HLH syndrome based on the 8 HLH-2004 criteria, excluding pHLH patients in the analysis did not alter the results, showing that in our cohort at least these patients could be diagnosed identically. Additionally, in contrast to a previous study, malignancy based sHLH patients could not be distinguished non-malignancy based sHLH.[21,40] This could be caused by the fact that we did not study every parameter separately, but rather the relation between the 8 criteria as a whole.

Limitations of our study warrant consideration. First, selection bias may have been introduced in this cohort, since the HLH-2004 criteria were used stringently to identify HLH patients (following current clinical practice), which may have led to underdiagnoses in this cohort, as borderline cases might have been classified as non-HLH. This could be prevented by using some of the newly proposed diagnostic tools (e.g. HScore, HLH in sJIA/SLE) and compare the outcomes with the values from the HLH-2004 criteria. This would also have enabled us to compare the performance of these scores and the newly proposed tool. Second, single stochastic regression imputation was used to complete missing data in the PCA dataset. This approach may have caused over-identification of interrelationships, as noise inherent to such datasets is reduced.[41] However, minimized this possible effect by replicating our results in a replication cohort. Even though this cohort contained more adults which had other underlying etiologies, the minimal parameter set could still identify HLH patients successfully. The specificity of ferritin, cytopenias and splenomegaly, as indicators of HLH, was lower in the replication cohort, suggesting that the non-HLH patients in this cohort were more similar to the HLH patients. This might be caused by a different approach in data inclusion or patient population. Third, although the dilution series have improved the diagnostic properties of the NK-lysis assay, there was a trend towards lower specificity in comparison with the traditional NK/lymphocyte function assay. However, in the context of the 8 HLH-2004 criteria, the introduction of the dilution series did not introduce false positive cases in our cohort.

In conclusion, we determined that specific combinations of the previously existent HLH-2004 criteria provide high specificity and sensitivity for a diagnosis of HLH syndrome. The minimal HLH parameter set identified here may improve outcome of HLH patients by facilitating rapid diagnosis of the HLH syndrome in patients undergoing evaluation. When confirmed in a prospective setting, this approach could be of value for timely diagnosis and treatment of the HLH syndrome.

Authorship Contributions

Contribution: B.M.S., J.M., C.L., L.C., and M.G. were involved in data collection in the UMCU; B.S. analyzed results and made the figures; A.V., C.B., F.A, R.M. and N.D. were responsible for data collection in the other hospitals. S.M. supplied the replication cohort from Johns Hopkins. B.S., J.J.B. and S.N. designed the research and wrote the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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SUPPLEMENTARY TABLES

Supplementary Table 1: The performance of splenomegaly, ferritin and cytopenia as screening criteria for HLH.

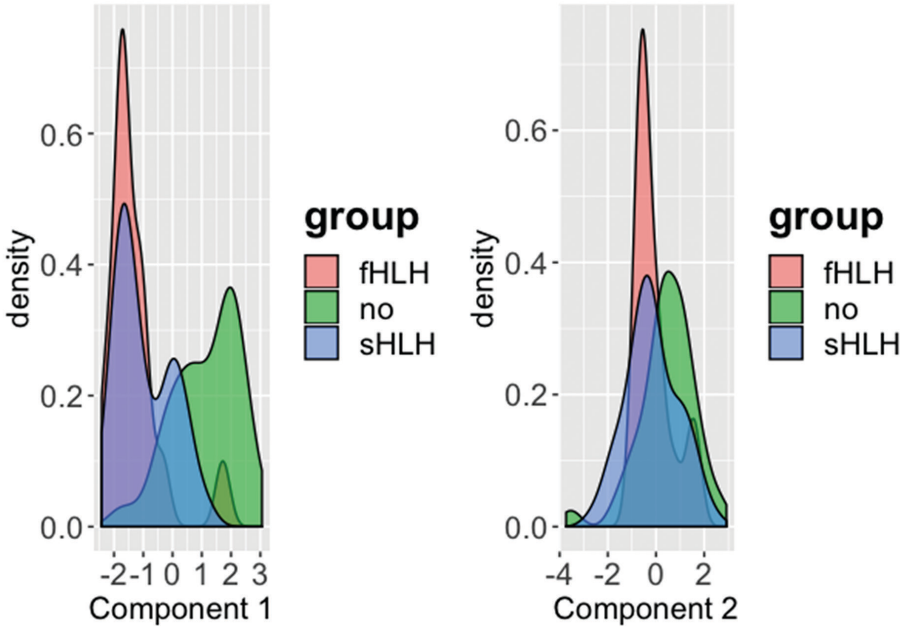
Test	Primary Sensitivity (CI)	Primary Specificity (CI)	Replicated Sensitivity (CI)	Replicated Specificity (CI)
1 Positive criterion	1.0 (0.96 – 1.0)	0.65 (0.56 – 0.74)	1.0 (0.97 – 1.0)	0.16 (0.06 – 0.31)
2 Positive criteria	0.77 (0.67 – 0.86)	0.92 (0.85 – 0.96)	0.98 (0.94 – 1.0)	0.45 (0.29 – 0.62)

Supplementary Table 2: Summary statistics on the patients in the replication cohort

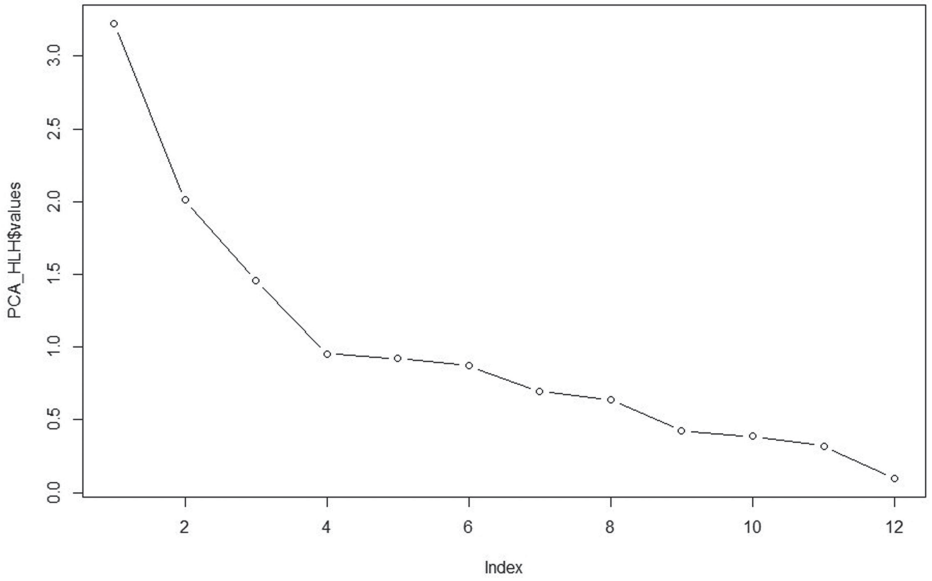
	non-HLH	sHLH
Number of Patients (n)	18	109
Median age (years)	54 (19 – 81)	58 (19 – 77)
Gender (% Male)	52.6	59.6
Type of Disease		
- Auto-Immune	18	15
- Immunodeficiency	0	0
- Genetic (non-HLH)	1	1
- Genetic (HLH)	0	0
- Infection	10	40
- Malignancy	5	43
- Unknown	1	9
- Other	3	0
Overall Survival (%)	71.1	38.5

SUPPLEMENTARY FIGURES

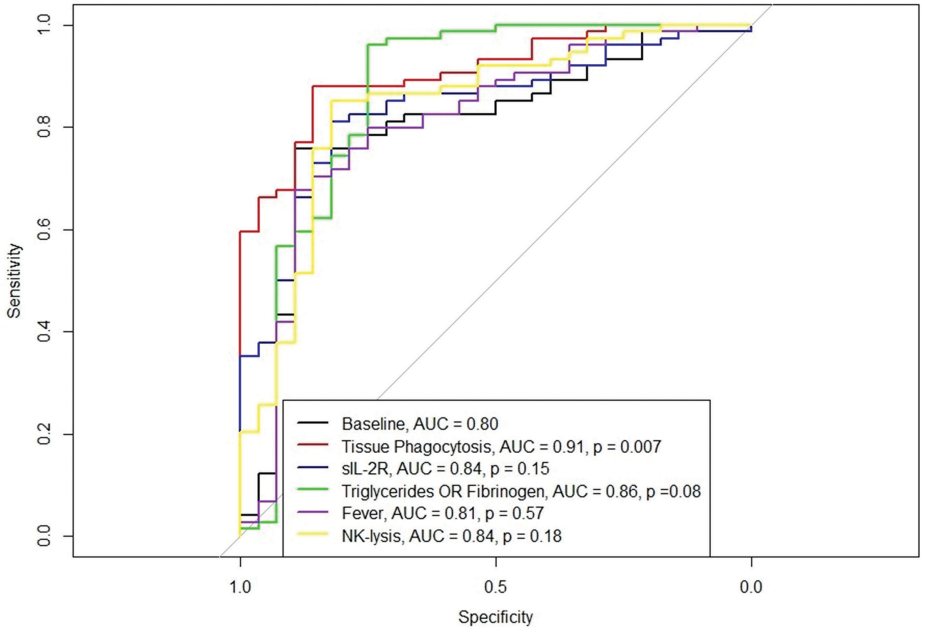
Supplementary Figure 1: Density plot of PLSDA analysis shows overlap between fHLH and sHLH patients.



Supplementary Figure 2: Elbow plot of principal component analysis shows that 4 components are sufficient for PCA.



Supplementary Figure 3: MultiROC analysis shows that the AUC of the diagnostic criteria is highest when Tissue Phagocytosis is added to the baseline model.



CHAPTER 12

General Discussion

Despite the state-of-the-art diagnostic evaluation and clinical care that are already applied in PAD, infectious sequelae and non-infectious complications still affect the majority of PAD patients.[1–3] These infectious sequelae and non-infectious complications greatly reduce the quality of life of PAD patients and cause severe morbidity and increased mortality.[2,3] Therefore, the aims of this thesis are to improve our understanding of the pathophysiology of (monogenetic) PAD and to identify new ways to improve management of infectious sequelae and non-infectious complications of PAD.

To this aim, we have chosen a translational approach in which we translate basic science to applied science ("bench-to-bedside"). Therefore, we used techniques in our *in vitro* assays that have already been implemented in clinical care and investigated hypotheses steered by clinical observations. We specifically focused on identifying new monogenetic variants that cause a PAD-like phenotype, immunomarkers that could serve as target for identification of non-infectious complications, and measures that could prevent bronchiectasis and standardized treatment for GLILD. We chose these subjects because they are some of the greatest challenges in PAD care and improving our understanding on these subjects could potentially directly improve the clinical care for PAD patients.[1,4–6] Therefore, we compared multiple national and international multicenter cohorts describing infectious sequelae and non-infectious complications in PAD. The chapters of this thesis are based on results reported by the Dutch National PID Study, the PAIRT Study and the STILPAD study. The National PID study is an ongoing retrospective and prospective observational cohort study investigating the epidemiology and pathophysiology of primary immunodeficiency in the Netherlands. In this study, all academic centers in the Netherlands prospectively collect demographic and clinical data and blood- and tissue samples of PAD patients and healthy controls during a follow-up period of 15-years. Moreover, retrospective clinical data that were generated before the start of the study are also collected.

The Prophylactic Antibiotics vs Immunoglobulin Replacement Therapy (PAIRT) study was a prospective randomized controlled open-label non-inferiority trial that compared the efficacy of prophylactic antibiotics and IRT in patients with IgSD and SPAD in a cross-over design. It involved all academic centers in the Netherlands, as well as the Jeroen Bosch Hospital, and was one of the first randomized controlled trials in PAD.[7]

The STudy of Interstitial Lung disease in Primary Antibody Deficiency (STILPAD) was an international retrospective and prospective observational trial that studied the epidemiology, pathophysiology and treatment of GLILD in CVID. Demographic, clinical and quality of life data on patients with GLILD was prospectively collected during a follow-up period of 5 years, as well as retrospective data that was acquired after GLILD onset but before the start of the study.

In this discussion, I will evaluate whether we have reached these aims and where opportunities for future research lie. Our previous findings will be discussed in the context of this thesis and recent publications as well as the clinical implications of these findings. Finally, I will sketch my perspective on future research topics of interest to the field.

T cells of PAD patients display features of dysregulation that can potentially be targeted with recently approved immunomodulators

This thesis sheds additional light on the pathophysiology of PAD. With the implementation of new next-generation sequencing techniques that have enabled clinical implementation of whole exome sequencing and even whole genome sequencing, the list of potential candidate genes involved in PAD is ever expanding.[8] It has been estimated that monogenetic causes of PAD are currently only identified in 15%-25% of patients who undergo NGS (panel) based genetic evaluation.[15] To improve clinical outcomes of PAD, it would be helpful if we could identify the underlying cause of PAD in all of our patients. However, previous research in monozygotic twins has shown that the majority of the variation in our immune system is determined by non-heritable factors.[16] Furthermore, PAD may develop at any age, suggesting that multifactorial and epigenetic causes that lead to a cumulative deterioration of B cell function might play a more important role in the pathophysiology of PAD.[17] We therefore not only focused on new candidate genes, but also on the immune (dys)function potentially involved in PAD pathogenesis:

- Dominant activating RAC2 variants cause impaired actin polymerization which leads to phagocyte, T and B cell dysfunction.
- Dominant negative DNA binding domain c-Myb variants cause T and B cell dysfunction through impaired transcription of genes involved in T and B cell maturation.
- Markers of T cell activation, exhaustion, homing and cytokine production can be used to distinguish between different monogenetic PAD associated diseases.

- Intestinal T cells might have a limited role in the pathophysiology of enteropathy in monogenetic PAD associated diseases.
- Inhibition of proliferation of effector/memory T cells of CVID patients by different types of immunosuppressive medication is limited, suggesting resistance to therapy.
- Sirolimus and ruxolitinib were more effective at inhibiting effector/memory T cell proliferation in CVID patients.
- Drugs that were effective at inhibiting proliferation, were also effective at reducing symptoms in individual patients.

Evidence presented on the two new variants is not yet sufficient to prove the causal relationship with PAD. [8] According to IUIS recommendations, variants should either have been reported in a pedigree consisting of multiple affected members or as de novo variants in multiple affected individuals. Moreover, functional molecular assays that support pathogenicity of new variants have to be designed and need to be replicated both in *in vitro* and *in vivo* systems. This is a necessary, but also complicated, time consuming and risky process and variants should therefore be carefully considered before they are subjected to analysis. Current prediction tools that have been implemented in routine clinical care are limited and do not yet adequately predict a variant's effect on protein structure, dynamics and protein-protein interactions. [15,16] New, easy to use, prediction tools that take these elements into account and could select new damaging, PAD associated variants with greater specificity should therefore be implemented.[17]

Aside from our efforts to unravel the role of immune (dys)function in PAD pathogenesis, recent efforts have revealed even more potential pathophysiological mechanisms of (non)-infectious complications in PAD. Berbers *et al.* have recently identified microbiota associated with airway disease and interstitial lung disease in XLA and CVID.[22] Moreover, they have also shown that non-infectious complications in CVID are associated with Th1 and Th17 activation.[23,24] Additionally, Strohmeier *et al.* and Unger *et al.* have shown that non-infectious complications and specifically enteropathy are associated with increased IFN-γ and upregulation of STAT.[21,25] Combined, these studies show interesting potential therapeutic targets for (non)-infectious complications in PAD, using recently improved immunomodulators like ustekinemab (Th17-inhibitor) and ruxolitinib (JAK/STAT-inhibitor).

A subgroup of PAD patients requires more intensive treatment and follow-up to properly manage infections and infectious complications.

Even though non-infectious complications are the most important cause of morbidity and mortality in PAD, infections and bronchiectasis have been found to attribute to the disease burden for 44%.[2] This thesis therefore elaborates on screening and management of infectious complications in PAD:

- Bronchiectasis does not progress in most PAD patients during follow-up.
- Patients with XLA, patients with recurrent infections and patients with pre-existent airway disease are at risk for progression of bronchiectasis.
- Prophylactic antibiotics and immunoglobulin replacement therapy are equally effective in preventing infections in IgSD and SPAD.
- Prophylactic antibiotics give less side effects, are easier to administer and less costly and should therefore be first-line treatments for IgSD and SPAD.
- Immunoglobulin replacement therapy could be effective in patients who have recurrent infections despite treatment with prophylactic antibiotics.

Combined, these studies show that some PAD patients require more intensive treatment to prevent infections and infectious sequelae like bronchiectasis. We show that that PAD patients who suffer frequent infections despite IRT or who have pre-existent bronchiectasis should be monitored more closely for progression of airway disease and that patients with milder forms of PAD that do not respond to prophylactic antibiotics should be managed with IRT instead. Patients who have recurrent infections despite IRT are potentially also of interest for more intensive interventions that could prevent infections and/or reduce bronchiectasis.

Corticosteroids could serve as first-line treatment for GLILD (and rituximab + azathioprine/MMF as second-line treatment).

GLILD has been shown to be one of the most important factors contributing to the increased mortality previously associated with non-infectious complications.[2] However, larger cohort studies that investigate optimal treatment for GLILD have been lacking. This thesis investigates the efficacy of frequently used treatment options for GLILD and provides evidence for recommendations regarding GLILD specific treatment:

- GLILD progresses in 44% - 68% of patients and leads to impaired pulmonary function and impaired blood oxygen levels.

- Hepatopathy, high ILD scores on baseline CT and high NK cell counts could be used to identify patients at risk for a poor clinical outcome.
- The reported efficacy of rituximab containing regimens is superior to that of corticosteroid therapy (75% - 95% vs 27%), although information regarding its safety is lacking.
- Few publications reported on the efficacy of corticosteroid therapy and the reported minimal efficacy is potentially subject to reporting bias.
- Therefore, the efficacy of corticosteroids was separately studied in a new observational cohort and corticosteroids were found to be effective in 42% of patients.
- Short-term corticosteroid treatment that only included induction therapy was equally effective as long-term treatment that included maintenance therapy.
- Short-term corticosteroid treatment showed minimal serious side effects.

Since short-term corticosteroids are able to maintain a long-lasting remission in almost half of the GLILD patients in this cohort and are found to have minimal serious side effects when used as short-term treatment, we propose that it should serve as first-line treatment and should be applied in patients at risk for a poor clinical outcome. Even though combination therapy with rituximab has been shown to be more effective, less is known about the safety of combination therapy in patients with PAD and rituximab is not always readily available to clinicians.[32] Moreover, combination therapy with rituximab has also been shown to be effective in patients in whom corticosteroids have failed to induce remission, making it a good candidate for second-line treatment.[32]

Clinical recommendations

The findings in this thesis, combined with recent findings by others in the field warrant the following clinical recommendations regarding PAD diagnosis, monitoring and treatment (Figure 4).

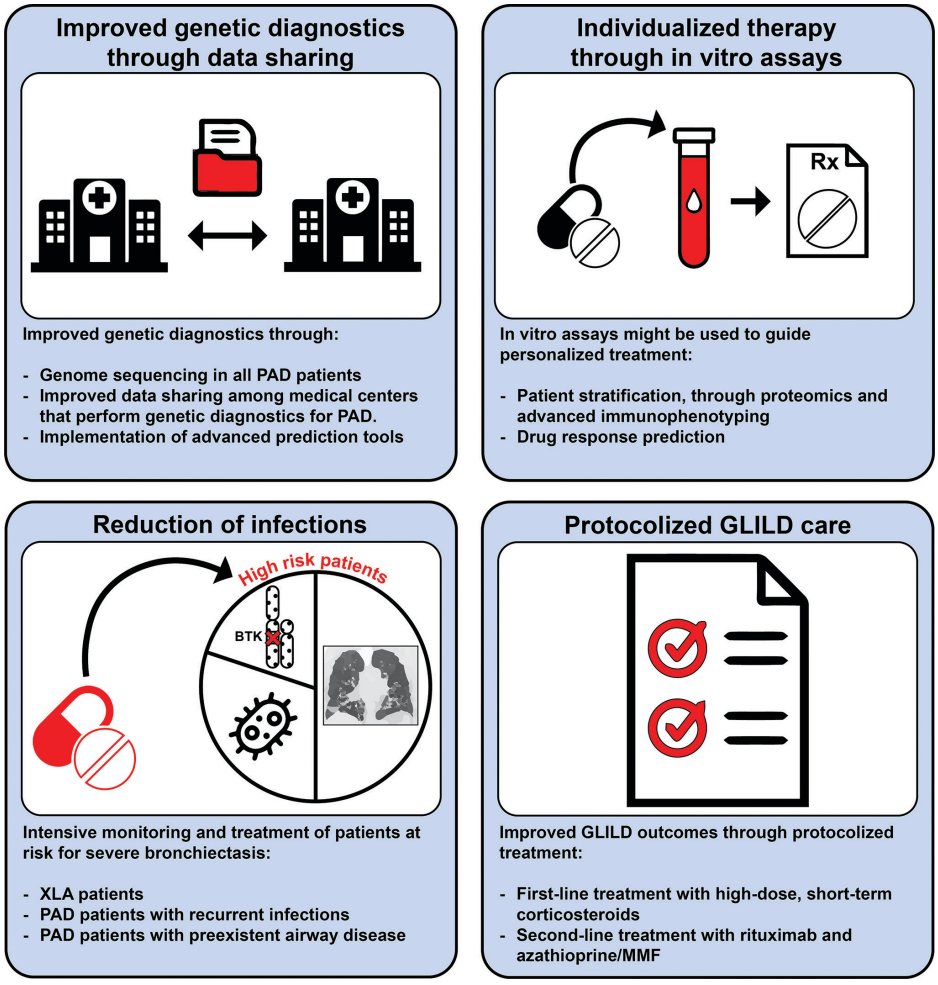
First, even though the diagnostic yield of NGS is limited, all PAD patients should be offered NGS based PID panel diagnostics. The clinical impact of a genetic diagnosis can be major and one study has reported a change in clinical management in 76% of the patients that received a genetic diagnosis.[6,33] Early PID panel diagnostics is already being implemented in the Netherlands and PID panel diagnostics are already offered to patients with inborn errors of immunity in multiple European countries. However, the types of inborn errors of immunity for which PID panel diagnostics are applied and the timing of PID panel diagnostics still varies widely

between clinicians. Therefore, the European Society of immunodeficiencies (ESID) genetics working party is currently preparing a guidance document for best practice regarding PID panel diagnostics in inborn error immunities.[34]

Additionally, an automated international platform that couples the finding of variants of unknown significance and (likely) pathogenic variants should be developed so that *de novo* variants affecting multiple individuals can be readily detected. Currently, a European platform where genetic variants specific for inborn errors of immunity and their clinical symptoms (ESID registry) can be reported is already in place. Moreover, international platforms that connect clinicians and researchers that have found genetic variants that are potentially clinically relevant, like GeneMatcher and MyGene2, also already exist.[35] However, all these platforms require active participation and active reporting, potentially introducing selection bias to the variants that are reported on these platforms. Instead, a platform that automatically collects potentially damaging *de novo* variants and automatically alerts clinicians and researchers when similar variants are found elsewhere could greatly speed-up research investigating new monogenetic causes of PAD. Not only would it enable researchers to find multiple affected individuals with greater ease, knowing the prevalence of potentially damaging *de novo* variants could also steer future research efforts and funding. However, protection of patient privacy and confidentially is a common issue in data sharing and verbal informed consent to data sharing is therefore a minimal requirement to be compliant to international regulations concerning patients' rights and privacy protection.[36,37]

Second, in vitro assays like those described in this thesis might help guide clinicians to select optimal individual treatment. PAD is a heterogeneous disease with a wide variety of potential infectious sequalae and non-infectious complications.[38] Due to this heterogeneity, an individualized therapeutical approach for PAD is often required.[1] Because PAD is such a rare disease, randomized clinical trials are often not feasible and therefore, immunomodulators that have been approved for the treatment of other auto-immune diseases are often not available for the treatment of non-infectious complications in PAD. By implementing the use of targeted proteomics and advanced immunophenotyping, we could identify potential responders to new immunomodulators, an approach that has already been shown to be effective in psoriatic arthritis.[39] Moreover, *in vitro* confirmation of the efficacy of these new immunomodulators could further enable compassionate use in PAD.

Figure 1: Visual summary of clinical recommendations formulated through the findings in this thesis and recent literature.



Third, patients at risk for recurrent infections and infectious sequelae should be monitored more intensively and possibly need prophylactic antibiotics and intensive IRT dosage with higher target trough levels.[40,41] Earlier research conducted in our cohort of PAD patients has shown that increased trough levels >10 g/L can potentially prevent the development of airway disease in CVID patients.[42] However, we could not replicate this finding in a later study, potentially due to the fact that target trough levels had already been increased during the follow-up study. Nonetheless, it could be of interest to raise target trough levels, especially in patients with preexistent bronchiectasis and in patients who still suffer from

recurrent infections, despite having trough levels >8 g/L. Furthermore, previous research has shown that preexistent bronchiectasis is especially prevalent among patients with a longer diagnostic delay.[2] This study has also shown that up until now efforts to reduce diagnostic delay have been in vain.[2] Future studies that investigate the use of predictive algorithms that can identify patients at risk for PAD in first-line care should be developed.

We also show that XLA patients are especially at risk for airway disease, as has also been previously reported in other studies.[43] Moreover, a small cohort study showed very poor outcome for XLA patients who had undergone a lung transplantation.[44] This begs the question whether a paradigm shift in the care for XLA patients is required and whether hematopoietic stem cell transplantation (HSCT) and gene editing should be investigated as treatment options for XLA patients at risk for severe airway disease.[45] In summary, more efforts are needed to prevent the development of bronchiectasis in our patients through early identification of PAD, optimal infection preventive treatment and potentially the introduction of HSCT and/or gene editing in XLA.

Finally, the morbidity and mortality that is caused by GLILD could be reduced by introducing standardized treatment, as proposed in this thesis. Moreover, patients at risk for disease progression and poor outcome should receive treatment as early as possible and for these patients we should abandon the watchful waiting approach, previously advocated by some clinicians.[31,46] Multiple studies show that specific interventions for GLILD fail to significantly improve pulmonary function tests, suggesting that function which has been lost cannot be regained.[32,46–48] Individualized, personalized treatment can be applied when standardized first-line and second-line treatment fail or if contraindications for first and/or second-line treatment exist. Standardized treatment regimens for GLILD could also enable observational research towards optimal treatment for GLILD. Future research towards treatment options for GLILD could mirror the research efforts that the HOVON consortium has performed for hematologic malignancies, by comparing new treatment regimens to historical treatment outcomes.[49] A registry that could accommodate this type of research should be developed specifically for GLILD.

Future perspectives

Despite these clinical recommendations there are still plenty of unanswered research questions that remain to be investigated in the case of PAD. Since PAD is a rare, heterogeneous disease, research containing well-described, well-controlled

quantitative outcomes is scarce. Future efforts to start and maintain multi-center, international collaborations, supported by experts in immunology, pulmonology, gastro-enterology, hematology, genetics and epidemiology should be undertaken. The observational cohorts generated by these types of efforts can be used for observational studies, but could also serve as historical controls for future research efforts. Moreover, randomized clinical trials are essential to achieve higher degrees of evidence regarding therapeutic interventions for infectious sequalae and non-infectious complications and are impossible to perform without such collaborations. Within international collaborations, randomized clinical trials that investigate interventions for PAD could be feasible depending on the anticipated incidence of the endpoint and the anticipated difference in effect size and should therefore be designed carefully.

Furthermore, the ex-vivo immunophenotyping and in vitro medication assays described in this thesis should be investigated further. First, we should further investigate the lack of correlation between blood and tissue subsets in the ex-vivo immunophenotyping assay and should analyze more patient samples, more types of tissue and both monogenetic and non-monogenetic PAD patients. Second, the in vitro medication assay should be further optimized in a prospective study. This way, standardized sampling of patients can be applied before immunomodulatory treatment is started and symptoms related to non-infectious complications can be quantified through the use of clinical scoring systems. Second, the assay could be expanded and used to test the efficacy of combination therapies. Combination therapy is frequently applied in the clinic to effectively target different parts or functions of the immune system.[50,51] Third, the assay could be altered so that extracellular targets like anti-TNF therapy and anti-CD20 therapy could be modelled. To this end, the assay could be performed in a 3D matrix instead of a 2D environment and should include phagocytes to mimic the antibody response. This approach has been previously used in multiple myeloma and bacterial infection models.[52,53] Another potential approach would be to use immunocompetent organoid models of patients with GLILD or enteropathy. This approach has previously been described for cancer and would be an interesting approach to increase our knowledge on the molecular processes that occur within the immune system at the tissue site of GLILD and enteropathy patients and how immunomodulators interact with these processes.[54] Downsides of organoid models, however, are their costs and scalability.

Moreover, new interventions that reduce infectious burden in PAD patients at risk for infectious sequelae should be investigated. First, a replication study of the major risk factor for bronchiectasis should be performed. Then, future interventions that aim to reduce infectious burden and/or bronchiectasis could be performed in this high-risk group. Interventions could consist of; 1) increasing the IRT dose and/or target IgG trough levels, 2) local supplementation of donor IgA antibodies, 3) standardized low-dose prophylactic antibiotics, 4) specific elimination of pathobionts in the microbiome and 5) HSCT or gene editing for high-risk XLA patients.

Finally, a first randomized clinical trial that compares treatment options for GLILD should be undertaken. Now that observational studies have estimated the efficacy of steroids and rituximab combination therapy separately, an RCT that compares the two treatment modalities directly is desperately needed. Previous observational studies did not use the same outcome measures, making direct comparison of effectivity difficult. Furthermore, safety has not yet been prospectively monitored for both regimens. Based on the outcomes of this future study, a more finite conclusion regarding the optimal first-line treatment of GLILD can be drawn.

Concluding remarks

This thesis discusses important pathophysiological mechanisms of (monogenetic) PAD as well as interventions that have the potential to reduce infectious burden, improve monitoring of bronchiectasis and reduce the amount of structural lung damage caused by GLILD. Efforts that further improve genetic diagnosis and that implement FACS assays which can identify therapeutic targets for non-infectious complications should be made. Moreover, an in vitro medication assay which can predict optimal treatment for non-infectious complications in clinical practice should be implemented, so that individualized treatment could be offered to patients that do not respond to standardized care.

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APPENDIX

SCIENTIFIC ENGLISH SUMMARY

Primary antibody deficiency

Primary antibody deficiencies (PAD) encompass various disorders characterized by reduced antibody production and are a common form of primary immunodeficiency. They include specific polysaccharide antibody deficiency (SPAD), immunoglobulin G subclass deficiency (IgSD), common variable immunodeficiency (CVID), and agammaglobulinemia (XLA). B cell dysfunction is a key feature of PAD, leading to recurrent respiratory and gastrointestinal infections due to compromised mucosal protection. Although preventive measures such as vaccination, prophylactic antibiotics, and immunoglobulin replacement therapy (IRT) have been effective in reducing infectious complications, some sequelae, including bronchiectasis, hearing loss, and malnutrition, still occur in PAD patients. Regular pulmonary screening is recommended, and further research is needed to optimize the use of IRT and prophylactic antibiotics in specific types of PAD and identify high-risk patients for bronchiectasis.

Non-infectious complications contribute significantly to the morbidity and mortality in CVID patients, despite receiving appropriate IRT. These complications affect multiple organ systems and include enteropathy, granulomatous lymphocytic interstitial lung disease (GLILD), and secondary hemophagocytic lymphohistiocytosis (HLH). Enteropathy, characterized by gastrointestinal inflammation, affects a significant proportion of CVID patients but lacks effective treatment options. GLILD, observed in a subset of CVID patients, leads to respiratory symptoms, and treatment guidelines are currently insufficient. HLH, a life-threatening immune dysregulation syndrome, can be triggered by severe infections or non-infectious complications in CVID patients, necessitating early intervention, although its diagnosis remains challenging due to non-specific criteria and delays in obtaining the necessary criteria.

Genetic studies have revealed that the genetic landscape of PAD is diverse, with over 40 genes identified as causative or associated with PAD using next-generation sequencing. These genes are involved in critical immunological signaling pathways and B cell development, highlighting the intricate interactions required for proper B cell maturation and immune function. However, monogenic causes are only identified in a small percentage of cases, underscoring the complex nature of PAD.

T cell dysfunction has also been implicated in PAD, extending beyond the B cell abnormalities typically associated with the condition. Specific monogenetic PAD-like diseases, as well as CVID patients without monogenetic disease experiencing non-infectious complications, demonstrate T cell dysregulation. Increased effector memory T cells and elevated expression of IFN-γ response genes have been observed in CVID patients with non-infectious complications. Additionally, dysregulated T cells and elevated IFN-γ production contribute to the activation of monocytes, leading to the production of B cell activating factor of the TNF family (BAFF). BAFF has been linked to the pathogenesis of non-infectious complications, particularly in GLILD patients.

To address the knowledge gaps in PAD diagnosis and management, this thesis aims to improve our understanding of the genetic landscape, identify optimal strategies for preventing infectious complications, and develop treatment approaches for non-infectious complications. The thesis highlights the need for larger cohort studies and randomized clinical trials to establish evidence-based recommendations. Additionally, identifying predictive risk factors for specific complications, such as bronchiectasis and progressive GLILD, is crucial for effective screening and treatment decisions. Given the role of T cell functionality in non-infectious complications, the thesis also focuses on exploring methods to normalize T cell function through clinical and preclinical assays.

Overall, the research conducted in this thesis strives to advance our knowledge of PAD, enhance our understanding of T cell dysfunction, and identify strategies to improve the management of infectious and non-infectious complications associated with PAD.

Part 1: Diagnosis and pathophysiology of PAD

The first part of this thesis aims to further unravel the hampered crosstalk between B and T cells in PAD by studying the functional T and B cell profiles of patients with a known monogenetic disease and patients with newly discovered genes potentially associated with PAD.

In chapter 2, we report on a new dominant activating variant in the RAC2 gene, RAC2-E62K, that leads to constitutively active RAC2. RAC2 plays an important role in actin polymerization in immune cells.[9] Remodeling of the actin skeleton is vital for important immune cell functions like cell division, phagocytosis and cell migration.[10] Previously, dominant negative mutations in RAC2 have been shown to mainly cause

phagocyte disorders.[11] Here, we and others use patient material, cellular models and a mouse model to show that the E62K variant causes constitutively active RAC2. Constitutively active RAC2 not only leads to a phagocyte disorder, but also to T cell and, potentially, B cell dysfunction.

In chapter 3, we report on new dominant negative mutations in the DNA binding domain of c-Myb, a transcription factor involved in T and B cell development.[13,14] This report discusses two patients who exhibited combined immunodeficiency-like symptoms and bone marrow failure. Both carried dominant negative mutations that potentially limit the DNA binding capacity of c-Myb, preventing transcription of important target genes and leading to T and B cell dysfunction. This study showed defective CD8+ cell signaling in one affected patient potentially caused by his specific c-Myb variant. This report provides the first evidence that c-Myb might potentially be a candidate gene for PAD.

In chapter 4, we compare the peripheral immune phenotype of primary immunoregulatory disorders (PIRD) subtypes associated with PAD-like disease and explore the intestinal immune phenotype. Peripheral T cell subsets were found to distinguish between PIRD subgroups, while intestinal T cells showed signs of limited infiltration, activation, and maturation. The study confirmed some previously observed differences in PIRD, but also showed conflicting results compared to previous studies, highlighting the variable immune phenotype within PIRD subgroups. Our findings show that peripheral T cells potentially play a vital role in PIRD and that their activation status, exhaustion status and homing status can potentially be used to distinguish between PIRD subtypes. Our findings also suggest that the intestinal T cell compartment may not be as important in intestinal pathology in PIRD as previously believed, suggesting the need for studying other intestinal immune subsets.

In chapter 5, we explore an in vitro T cell assay for predicting individual treatment responses in common variable immunodeficiency with complications (CVIDc). The results show that T cell proliferation induced by aCD3 stimulation can be inhibited by immunosuppressants, and the effectiveness of inhibition differs among CVID subgroups. Intracellular cytokine production, however, was not a reliable predictor of clinical response. Our results suggest that combined T cell subset proliferation could moderately predict clinical response.

Part 2: Management of PAD

The second part of this thesis aims to give new insights in the prevention of infectious complications and the management of non-infectious complications in PAD. To this aim we used retrospective, prospective and randomized clinical studies to further elucidate risk factors associated with a poor outcome of infectious and non-infectious-complications and compare effectivity of treatment regimens that prevent infectious and non-infectious-complications.

In chapter 6, we study potential risk factors for airway disease and airway disease related bronchiectasis in patients with PAD that are treated with IRT. Here we find that airway disease and airway disease related bronchiectasis are stable in most patients during a follow-up of ten years. This study also shows that patients with XLA, patients with recurrent infections and patients with pre-existent airway disease are more at risk for progression of airway disease. Prophylactic antibiotics were potentially given more frequently to patients with airway disease progression, but did not manage to halt the progression.

In chapter 7, we compare the efficacy and safety of prophylactic antibiotics and immunoglobulin replacement therapy (IRT) in patients with IgSD and SPAD. Both treatment regimens have previously been reported to be effective in these milder forms of PAD.[26] In this study these treatment regimens were equally effective. Moreover, patients treated with prophylactic antibiotics suffered less adverse events and prophylactic antibiotics are easier to administer and less costly. Based on these findings, we recommend prophylactic antibiotics as first-line treatment for these milder forms of PAD. Importantly, we also found that a subgroup of patients still suffered from recurrent infections, despite treatment with prophylactic antibiotics. We found that IRT could reduce the infectious burden in these patients. We could not identify other clinical characteristics that could distinguish patients with milder forms of PAD that did not respond to prophylactic antibiotics.

In chapter 8, we elaborate on clinical signs, symptoms, and the natural course of granulomatous-lymphocytic interstitial lung disease (GLILD). The study confirms that GLILD is associated with an increased female/male ratio, splenomegaly, lymphadenopathy, auto-immune cytopenia, hepatopathy, enteropathy, reduced switched memory B cells, increased $CD21^{low}$ B cells, and reduced CD4+ naive cells. Without treatment, the majority of GLILD patients experience progression. A poor clinical outcome, defined as severely impaired pulmonary function tests, pulmonary hypertension, or oxygen dependency, was observed in 47% of

patients. Hepatopathy, high ILD scores on baseline CT, and high NK cell counts can potentially serve as clinical markers to identify patients at risk for a poor clinical outcome, guiding early treatment initiation.

In chapter 9 we provide a systematic review of the existing evidence on treatment options for GLILD. The majority of evidence consists of case reports and small cohort studies, with a lack of well-controlled studies with quantitative outcome measures. However, studies have reported qualitative or quantitative outcomes for treatment with corticosteroids, rituximab monotherapy, and rituximab combined with azathioprine or mycophenolate mofetil. Rituximab, both as monotherapy and in combination, showed qualitative improvements in pulmonary function tests, radiological findings, and clinical symptoms in 75% to 95% of cases. Combination therapy appeared to be more effective, with more frequent reporting in the literature. Corticosteroid therapy showed limited efficacy (27%), possibly due to reporting bias and the assumption of its effectiveness as first-line treatment.

In chapter 10 we focus on the efficacy and safety of corticosteroids as a first-line treatment for GLILD. This study shows that corticosteroids can induce a long-lasting remission in 42% of patients, and side effects and infections were primarily associated with prolonged exposure to corticosteroids during maintenance therapy. Short-term treatment, consisting of an induction phase and tapering phase, was equally effective in maintaining remission as long-term therapy with maintenance therapy. Given the ability of short-term corticosteroid treatment to maintain long-lasting remission with minimal serious side effects, the study proposes it as the first-line treatment. Combination therapy with rituximab, although more effective, has less known safety data and is considered a good candidate for second-line treatment when corticosteroids fail.

In chapter 11, we used a retrospective analysis of a discovery and a validation cohort, to assess the diagnostic value of the HLH-2004 criteria for early identification of the HLH syndrome. By utilizing statistical methods such as hierarchical cluster analysis and principal component analysis, this study identified a parameter set that could exclude HLH syndrome and a minimal parameter set consisting of major and minor criteria for positive identification. The minimal parameter set, including two major criteria (hemophagocytosis and splenomegaly) and three minor criteria (cytopenia, increased ferritin, and increased triglycerides/low fibrinogen), predicted HLH with high sensitivity (95%) and specificity (94%).

Implementing this approach could improve patient outcomes by enabling rapid diagnosis and treatment of HLH syndrome.

Clinical recommendations

In this thesis, we present important clinical recommendations for the diagnosis, monitoring, and treatment of primary antibody deficiencies (PAD). Based on our findings and recent research in the field, we propose several key recommendations.

Firstly, we recommend that all PAD patients should be offered next-generation sequencing (NGS) based panel diagnostics to improve the diagnostic yield. While NGS has its limitations, a genetic diagnosis can have a significant clinical impact, leading to changes in management for a majority of patients. To facilitate the identification of relevant genetic variants affecting multiple individuals, we propose the development of an automated international platform that connects clinicians and researchers. This platform would allow for the efficient sharing and detection of variants of unknown significance and (likely) pathogenic variants, accelerating research on new monogenetic causes of PAD.

Secondly, we emphasize the heterogeneity of PAD and the need for individualized treatment approaches. To aid clinicians in selecting optimal treatments, we suggest the use of in vitro assays such as targeted proteomics and advanced immunophenotyping. These assays can help us identify potential responders to new immunomodulators, allowing for personalized treatment options. Additionally, they can be used to confirm the efficacy of these immunomodulators, enabling their compassionate use in PAD patients.

Thirdly, we highlight the importance of intensive monitoring and proactive measures for patients at risk of recurrent infections and infectious complications. This may include considering prophylactic antibiotics and higher target trough levels of immunoglobulin replacement therapy (IRT). Patients with preexistent bronchiectasis and ongoing infections, despite adequate trough levels, may benefit from further raising the target trough levels. Furthermore, we believe that efforts should be made to reduce the diagnostic delay of PAD, including the development of predictive algorithms that can identify high-risk patients in first-line care.

In our research, we find that X-linked agammaglobulinemia (XLA) patients are particularly susceptible to airway disease. We propose exploring alternative

treatment options such as hematopoietic stem cell transplantation (HSCT) and gene editing for XLA patients at risk of severe airway disease.

Finally, we advocate for standardized treatment approaches for granulomatous-lymphocytic interstitial lung disease (GLILD) to reduce its associated morbidity and mortality. Early treatment initiation is crucial for patients at risk of disease progression, and individualized treatment options can be considered when standard approaches fail.

Future perspectives and final conclusion

While providing these clinical recommendations, we acknowledge that there are still many unanswered research questions in the field of PAD. We emphasize the need for collaborative efforts, including multi-center, international collaborations involving experts in various disciplines. These collaborations would facilitate observational studies and randomized clinical trials, which are essential for obtaining higher degrees of evidence for therapeutic interventions in PAD.

In conclusion, this thesis sheds light on the pathophysiological mechanisms of PAD and proposes recommendations to improve diagnosis, monitoring, and treatment. By implementing genetic diagnostics, utilizing in vitro assays for personalized treatment, and ensuring intensive monitoring and proactive measures, we can enhance the care provided to PAD patients. However, further research and collaborative efforts are necessary to address remaining knowledge gaps and advance our understanding and management of this complex disease.

WETENSCHAPPELIJKE NEDERLANDSE SAMENVATTING

Primaire antilichaamdeficiëntie

Primaire antilichaamdeficiëntie (PAD) omvat verschillende aandoeningen waarbij te weinig antilichamen worden aangemaakt, en het is de meest voorkomende vorm van primaire immuundeficiëntie. Enkele voorbeelden van PAD zijn specifieke polysaccharide-antilichaamdeficiëntie (SPAD), immunoglobuline G-subklassen deficiëntie (IgSD), common variabele immunodeficiëntie (CVID) en agammaglobulinemie (XLA). PAD ontstaat doordat de B-cellen niet goed functioneren, waardoor de slijmvliezen onvoldoende beschermd zijn en herhaaldelijke infecties aan de luchtwegen en het maag-darmkanaal optreden. Hoewel preventieve maatregelen zoals vaccinatie, antibiotica en behandeling met donor antilichamen (IRT) helpen bij het verminderen van infecties, treden er nog steeds complicaties zoals bronchiëctasieën, gehoorverlies en ondervoeding op bij mensen met PAD. Het is belangrijk om regelmatig longcontroles uit te voeren en meer onderzoek te doen naar de beste behandelingen en risicofactoren voor bronchiëctasieën.

Sinds de introductie van IRT zijn niet-infectieuze complicaties de belangrijkste oorzaak van gezondheidsproblemen en sterfte bij CVID-patiënten. Deze complicaties omvatten enteropathie (ontsteking van de darmen), granulomateuze lymfocytaire interstitiële longziekte (GLILD) en hemofagocytaire lymfohistiocytose (HLH), een zeldzame en gevaarlijke immuunstoornis. Een aanzienlijk aantal CVID-patiënten ontwikkelt enteropathie, maar er zijn momenteel geen effectieve behandelingsopties beschikbaar. GLILD veroorzaakt ademhalingsproblemen bij sommige CVID-patiënten, maar er zijn nog onvoldoende richtlijnen voor de behandeling ervan. HLH is een levensbedreigende aandoening die kan ontstaan bij CVID-patiënten als gevolg van ernstige infecties of niet-infectieuze complicaties. Snelle diagnose is cruciaal, maar het is moeilijk vanwege de niet-specifieke criteria en de tijd die nodig is om aan de vereiste criteria te voldoen.

Uit studies is gebleken dat meer dan 40 genen betrokken zijn bij PAD. Deze genen spelen een belangrijke rol in het immuunsysteem, met name bij de ontwikkeling van B-cellen. Dit wijst op de complexe interacties die nodig zijn voor een goede rijping van B-cellen en een goede immuunfunctie. Desondanks worden monogenetische oorzaken slechts in een klein percentage van de gevallen vastgesteld, wat de complexiteit van PAD benadrukt.

Eerdere onderzoeken hebben aangetoond dat PAD ook gepaard kan gaan met verstoorde T-celfunctie. Stoornissen in de regulatie van T-cellen zijn waargenomen bij specifieke monogenetische vormen van PAD en bij CVID-patiënten met niet-infectieuze complicaties. CVID-patiënten met niet-infectieuze complicaties vertonen meer geheugen T-cellen en een verhoogde productie van IFN-γ, wat een rol speelt bij de activatie van monocyten en de productie van de B-celactiverende factor (BAFF). BAFF is in verband gebracht met het ontstaan van niet-infectieuze complicaties, met name bij GLILD-patiënten.

Het doel van dit onderzoek is het opvullen van de kennislacunes op het gebied van de diagnose en behandeling van PAD. Het richt zich op het verbeteren van ons begrip van de genetische aspecten van PAD, het identificeren van optimale strategieën om infectiecomplicaties te voorkomen en behandelplannen te ontwikkelen voor niet-infectieuze complicaties. Grootschalige onderzoeken en klinische studies worden uitgevoerd om op bewijs gebaseerde aanbevelingen te kunnen doen. Daarnaast worden voorspellende risicofactoren onderzocht voor specifieke complicaties zoals bronchiëctasieën en progressieve GLILD, zodat screening effectiever wordt en behandeling eerder ingezet kan worden. Gezien de rol van T-cellen bij niet-infectieuze complicaties, wordt ook onderzocht hoe de functie van T-cellen genormaliseerd kan worden. Kortom, dit onderzoek heeft tot doel onze kennis van PAD te vergroten, ons begrip van T-celfunctie in PAD te verbeteren en strategieën te identificeren om zowel infectieuze als niet-infectieuze complicaties bij PAD beter te kunnen behandelen.

Deel 1: Diagnose en pathofysiologie van primaire antilichaamdeficiëntie (PAD)
Het eerste deel van dit proefschrift is gericht op het verder ontrafelen van het verstoorde samenspel tussen B- en T-cellen bij PAD. Dit wordt gedaan door de functionele profielen van T- en B-cellen te bestuderen bij patiënten met bekende monogenetische ziekten en patiënten waarbij nieuw ontdekte genen mogelijk geassocieerd worden met PAD.

In hoofdstuk 2 wordt gerapporteerd over een nieuwe dominante activerende variant in het RAC2-gen, genaamd RAC2-E62K, die leidt tot constitutief actief RAC2. RAC2 speelt een belangrijke rol bij actinepolymerisatie in immuuncellen, wat essentieel is voor belangrijke functies zoals celdeling, fagocytose en celmigratie. Eerdere studies toonden aan dat dominante negatieve mutaties in RAC2 normale fagocyt functie verstoren. In dit onderzoek wordt aangetoond dat de E62K-variant

constitutief actief RAC2 veroorzaakt, wat niet alleen resulteert in verstoorde fagocyt functie, maar mogelijk ook in verstoorde T- en B-cel functie.

In hoofdstuk 3 worden nieuwe dominante negatieve mutaties in het DNA-bindende domein van c-Myb besproken, een transcriptiefactor die betrokken is bij de ontwikkeling van T- en B-cellen. Deze studie richt zich op twee patiënten met T- en B-cel dysfunctie en beenmergfalen. Beide patiënten hadden dominante negatieve mutaties in het DNA-bindende domein van c-Myb, wat mogelijk deze T- en B-cel dysfunctie verklaarde. Celkweek met cellen van één patiënt toonde een defecte CD8+ cel signalering aan in c-Myb. Dit suggereert dat c-Myb een kandidaatgen is voor PAD.

Hoofdstuk 4 richt zich op het vergelijken van het perifere immuunfenotype van subtypen van primaire immunoregulatoire aandoeningen (PIRD) die geassocieerd zijn met PAD-achtige ziekten, en onderzoekt het immuunfenotype van de darm. Het onderzoek identificeerde perifere T-celsubsets die onderscheid kunnen maken tussen verschillende PIRD-subgroepen, terwijl darm-T-cellen tekenen vertoonden van beperkte infiltratie, activatie en rijping. Deze bevindingen benadrukken het variabele immuunfenotype binnen PIRD-subgroepen. Het suggereert dat perifere T-cellen mogelijk een belangrijke rol spelen bij PIRD en dat hun activatiestatus, uitputtingsstatus en migratiestatus kunnen helpen bij het onderscheiden van PIRD-subtypen. Bovendien wijst het onderzoek erop dat het darm-T-celcompartiment mogelijk niet zo cruciaal is bij PIRD-gerelateerde darmziekten als voorheen gedacht, en dat toekomstige studies andere immuunsubsets in de darm moeten onderzoeken.

In hoofdstuk 5 wordt een in vitro T-cel-assay onderzocht om individuele behandelingsreacties te voorspellen bij CVID met niet-infectieuze complicaties (CVIDc). De resultaten tonen aan dat T-celproliferatie, geïnduceerd door aCD3-stimulatie, geremd kan worden door immunosuppressiva, waarbij de effectiviteit van remming verschilt tussen verschillende CVID-subgroepen. Intracellulaire cytokineproductie bleek echter geen betrouwbare voorspeller te zijn van klinische respons. Deze bevindingen suggereren dat de gecombineerde proliferatie van T-celsubsets mogelijk een voorspeller kan zijn van klinische respons.

Deel 2: Behandeling van PAD

Het tweede deel van dit proefschrift heeft als doel nieuwe inzichten te geven in de preventie en de behandeling van infectieuze complicaties en niet-infectieuze

complicaties bij PAD. Hiervoor hebben we retrospectieve, prospectieve en gerandomiseerde klinische studies gebruikt om risicofactoren te vinden die geassocieerd zijn met een slechte uitkomst van infectieuze en niet-infectieuze complicaties, en om de effectiviteit van behandelingsregimes die infectieuze en niet-infectieuze complicaties voorkomen te vergelijken.

In hoofdstuk 6 bestuderen we mogelijke risicofactoren voor bronchiëctasieën bij patiënten met PAD die met IRT worden behandeld. Hieruit blijkt dat bronchiëctasieën bij de meeste patiënten stabiel blijven gedurende een follow-up van tien jaar. Deze studie laat ook zien dat patiënten met XLA, patiënten met terugkerende infecties en patiënten met reeds bestaande luchtwegaandoeningen een groter risico lopen op progressie van luchtwegaandoeningen. Profylactische antibiotica werden mogelijk vaker gegeven aan patiënten met progressie van luchtwegaandoeningen, maar slaagden er niet in de progressie te stoppen.

In hoofdstuk 7 vergelijken we de effectiviteit en veiligheid van profylactische antibiotica en IRT bij patiënten met IgSD en SPAD. Beide behandelingsregimes zijn eerder effectief gebleken bij deze mildere vormen van PAD. In deze studie waren beide behandelingsregimes even effectief. Bovendien hadden patiënten die met profylactische antibiotica werden behandeld minder bijwerkingen en zijn profylactische antibiotica gemakkelijker toe te dienen en minder kostbaar. Op basis van deze bevindingen raden we profylactische antibiotica aan als eerstelijnstherapie voor deze mildere vormen van PAD. Belangrijk is dat we ook hebben ontdekt dat een subgroep van patiënten nog steeds last had van terugkerende infecties, ondanks de behandeling met profylactische antibiotica. We ontdekten dat IRT de infectieuze druk bij deze patiënten kon verminderen. We konden geen andere klinische kenmerken identificeren die patiënten met mildere vormen van PAD die niet reageerden op profylactische antibiotica konden onderscheiden.

In hoofdstuk 8 gaan we dieper in op klinische tekenen, symptomen en het natuurlijke beloop van GLILD. De studie bevestigt dat GLILD geassocieerd is met een verhoogde vrouw/man-verhouding, splenomegalie, lymfadenopathie, auto-immuuncytopenie, hepatopathie, enteropathie, verminderde switched memory B-cellen, verhoogde CD21low B-cellen en verminderde CD4+ naïeve cellen. Zonder behandeling ervaart de meerderheid van de GLILD-patiënten progressie. Een slechte klinische uitkomst, gedefinieerd als ernstig verminderde longfunctietests, pulmonale hypertensie of zuurstofafhankelijkheid, werd waargenomen bij 47%

van de patiënten. Hepatopathie, hoge ILD-scores op baseline CT en een hoog aantal NK-cellen kunnen potentieel dienen als klinische markers om patiënten met een slechte klinische uitkomst te identificeren en deze vroegtijdig te behandelen.

In hoofdstuk 9 geven we een systematische review van het bestaande bewijs over behandelingsmogelijkheden voor GLILD. Het merendeel van het bewijs bestaat uit casusrapporten en kleine cohortstudies en er was een gebrek aan goed gecontroleerde studies met kwantitatieve uitkomstmaten. Er waren echter studies die kwalitatieve of kwantitatieve resultaten hadden gerapporteerd voor behandeling met corticosteroïden, rituximab-monotherapie en rituximab in combinatie met azathioprine of mycofenolaatmofetil. Rituximab, zowel als monotherapie als in combinatie, vertoonde kwalitatieve verbeteringen in longfunctietests, radiologische bevindingen en klinische symptomen bij 75% tot 95% van de gevallen. Corticosteroïdtherapie vertoonde beperkte werkzaamheid (27%), mogelijk door rapportagebias, omdat effectiviteit ervan als eerstelijnstherapie voor lief wordt genomen.

In hoofdstuk 10 richten we ons op de werkzaamheid en veiligheid van corticosteroïden als eerstelijnstherapie voor GLILD. Deze studie toont aan dat corticosteroïden een langdurige remissie kunnen induceren bij 42% van de patiënten, en bijwerkingen en infecties waren voornamelijk geassocieerd met langdurige blootstelling aan corticosteroïden tijdens onderhoudstherapie. Kortdurende behandeling, bestaande uit een inductiefase en afbouwfase, was even effectief in het behouden van remissie als langetermijntherapie met onderhoudstherapie. Gezien het vermogen van kortdurende corticosteroïdbehandeling om langdurige remissie te handhaven met minimale ernstige bijwerkingen, wordt het als eerstelijnstherapie voorgesteld. Ondanks dat ombinatietherapie met rituximab effectiever lijkt, zijn er minder gegevens bekend over de veiligheid en wordt daarom beschouwd als een goede kandidaat voor tweedelijnstherapie wanneer corticosteroïdentherapie faalt.

In hoofdstuk 11 hebben we een retrospectieve analyse uitgevoerd van een ontdekkings- en validatiecohort om de diagnostische waarde van de HLH-2004-criteria voor vroege identificatie van het HLH-syndroom te beoordelen. Door statistische methoden zoals hiërarchische clusteranalyse en principale componentenanalyse te gebruiken, heeft deze studie een parameter set geïdentificeerd die het HLH-syndroom kan uitsluiten, en een minimale parameter set voor positieve identificatie. De minimale parameter set, inclusief

twee belangrijke criteria (hemofagocytose en splenomegalie) en drie minder belangrijke criteria (cytopenie, verhoogd ferritine en verhoogde triglyceriden/ laag fibrinogeen), voorspelde HLH met een hoge gevoeligheid (95%) en specificiteit (94%). Implementatie van deze aanpak kan de resultaten voor patiënten verbeteren door een snelle diagnose en behandeling van het HLH-syndroom mogelijk te maken.

Klinische aanbevelingen
In deze scriptie presenteren we belangrijke klinische aanbevelingen voor de diagnose, monitoring en behandeling van primaire antilichaamdeficiënties (PAD). Op basis van onze bevindingen en recent onderzoek op dit gebied stellen we verschillende belangrijke aanbevelingen voor.

Ten eerste raden we aan dat alle PAD-patiënten next-generation sequencing (NGS) aangeboden krijgen. Hoewel NGS zijn beperkingen heeft, kan een genetische diagnose een aanzienlijke klinische impact hebben, wat leidt tot veranderingen in de behandeling voor de meerderheid van de patiënten. Om de identificatie van relevante genetische varianten te vergemakkelijken, stellen we de ontwikkeling voor van een geautomatiseerd internationaal platform dat clinici en onderzoekers met elkaar verbindt. Dit platform zou het mogelijk maken genetische varianten efficiënter te detecteren en te delen, wat het onderzoek naar nieuwe monogenetische oorzaken van PAD zou versnellen.

Ten tweede benadrukken we de heterogeniteit van PAD en de noodzaak van gepersonaliseerde behandelaanpakken. Om clinici te helpen bij het selecteren van optimale behandelingen, suggereren we het gebruik van in vitro testen zoals gerichte proteomics en geavanceerde immunofenotypering. Deze testen kunnen helpen bij het identificeren van patiënten die goed reageren op nieuwe immunomodulatoren, waardoor gepersonaliseerde behandelopties mogelijk worden. Bovendien kunnen ze worden gebruikt om de werkzaamheid van deze immunomodulatoren te bevestigen, waardoor behandeling bij PAD-patiënten mogelijk wordt.

Ten derde benadrukken we het belang van intensieve monitoring en behandeling voor patiënten met een verhoogd risico op terugkerende infecties en infectieuze complicaties. Dit kan bestaan uit profylactische antibiotica of hogere streefwaarden van IRT. Patiënten met reeds bestaande bronchiëctasieën en aanhoudende infecties, ondanks adequate streefwaarden, kunnen baat hebben bij verdere

verhoging van de streefwaarden. Bovendien geloven we dat inspanningen moeten worden geleverd om de diagnostische vertraging van PAD te verminderen, inclusief de ontwikkeling van voorspellende algoritmen die hoog risicopatiënten in de eerstelijnszorg kunnen identificeren.

In ons onderzoek vinden we dat patiënten met XLA bijzonder vatbaar zijn voor luchtwegaandoeningen. We stellen voor om alternatieve behandelingsopties te onderzoeken, zoals hematopoëtische stamceltransplantatie en gentherapie, voor XLA-patiënten met een hoog risico op ernstige luchtwegaandoeningen.

Tot slot pleiten we voor gestandaardiseerde behandeling van GLILD om de daarmee gepaard gaande morbiditeit en mortaliteit te verminderen. Vroegtijdige behandeling is cruciaal voor patiënten met een risico op ziekteprogressie, en individuele behandelopties kunnen worden overwogen wanneer standaardbenaderingen falen.

Toekomstperspectieven en conclusie

Ondanks deze klinische aanbevelingen, erkennen we dat er nog veel onbeantwoorde onderzoeksvragen zijn op het gebied van PAD. We benadrukken de noodzaak van samenwerkingsverbanden, waaronder multicenter, internationale samenwerkingen met experts uit verschillende disciplines. Deze samenwerkingen zouden observationele studies en gerandomiseerde klinische onderzoeken vergemakkelijken, die essentieel zijn om hogere niveaus van bewijs te verkrijgen voor therapeutische interventies bij PAD.

Concluderend schijnt deze thesis licht op de pathofysiologische mechanismen van PAD en stelt aanbevelingen voor om de diagnose, monitoring en behandeling te verbeteren. Door genetische diagnostiek te implementeren, in vitro testen te gebruiken voor gepersonaliseerde behandeling en intensieve monitoring en proactieve maatregelen te waarborgen, kunnen we de zorg voor PAD-patiënten verbeteren. Verdere onderzoek en samenwerkingsinspanningen zijn echter noodzakelijk om resterende kennishiaten aan te pakken en ons begrip en beheer van deze complexe ziekte te bevorderen.

NEDERLANDSE SAMENVATTING (VOOR NIET INGEWIJDEN)

Het afweersysteem

Het afweersysteem is een ingewikkeld netwerk van gespecialiseerde cellen, ook wel witte bloedcellen genoemd, en eiwitten. Samen, houdt dit netwerk ziekteverwekkers als bacteriën en virussen buiten de deur. Daarnaast ruimt het afweersysteem ook dode cellen en andere rommel in het lichaam op en kan het zelfs vroege stadia van kanker herkennen en opruimen. De witte bloedcellen bestaan uit een aantal verschillende groepen, granulocyten, macrofagen en lymfocyten. In deze thesis zullen de lymfocyten worden besproken. De lymfocyten bestaan uit B-cellen, T-cellen en NK-cellen. B-cellen spelen een belangrijke rol in de afweer in ons bloed en in onze slijmvliezen. Nadat een B-cel een ziekteverwekker herkend heeft kan de cel antilichamen produceren, wat eiwitten zijn die heel nauwkeurig aan een ziekteverwekker kunnen binden en ziekteverwekkers zo onschadelijk maken. B-cellen kunnen dit langdurig blijven doen, waardoor de afweer geheugen tegen verschillende ziekteverwekkers kan ontwikkelen. Deze strategie wordt ook ingezet bij vaccinatie, waarbij we een klein stukje ziekteverwekker inspuiten zodat de B-cellen dit herkennen en antilichamen gaan produceren.

T-cellen kunnen cellen herkennen die geïnfecteerd zijn geraakt met een ziekteverwekker en vervolgens deze cel doden of signaalstoffen rondsturen die andere afweercellen aantrekt om deze cel te doden. NK-cellen of natural killer cellen scannen cellen op foutjes die kunnen ontstaan door een virusinfectie of een beginnende kanker en maken cellen die deze foutjes laten zien dood.

Primaire antilichaamstoornissen

Primaire afweerstoornissen zijn ziektes waarbij onderdelen van de afweer het niet meer goed doen. Bij primaire antilichaamstoornissen werken de B-cellen niet goed genoeg, waardoor deze minder of helemaal geen antilichamen produceren. Dit leidt tot herhaalde infecties en andere gezondheidsproblemen. Primaire antilichaamstoornissen kunnen erfelijk zijn en worden dan veroorzaakt door veranderingen in verschillende genen.

Dankzij nieuwe onderzoekstechnieken zijn er steeds meer genetische varianten ontdekt die verband houden met primaire antilichaamstoornissen. Op dit moment kennen we meer dan 40 genen die erfelijke primaire antilichaamstoornissen kunnen veroorzaken. Toch kunnen we slechts bij 10-20% van de patiënten een erfelijke oorzaak vinden, terwijl het vinden van een genetische oorzaak grote

impact kan hebben op de behandelmogelijkheden voor de individuele patiënt. Daarom is het belangrijk om de erfelijkheid van primaire antilichaamstoornissen nog beter te begrijpen.

Primaire antilichaamstoornissen kunnen verschillende organen aantasten omdat er naast infecties ook niet-infectieuze complicaties kunnen optreden. T-cellen, een ander type afweercel, spelen een belangrijke rol bij deze complicaties. Het wordt verondersteld dat T-cellen die gericht zijn tegen het eigen lichaam, de organen van patiënten beschadigen. De darm en de longen zijn de organen die hierdoor het vaakst worden aangetast. Wanneer T-cellen de darm aantasten noemen we dat enteropathie en wanneer ze de longen aantasten noemen we dat GLILD[1]. Niet-infectieuze complicaties kunnen ernstige gevolgen hebben en moeten daarom vroegtijdig worden opgespoord en behandeld.

Het doel van dit onderzoek is om een beter begrip te krijgen van de erfelijke en niet-erfelijke oorzaken van primaire antilichaamstoornissen. We willen wetenschappelijk bewijs leveren dat kan helpen bij de behandeling van zowel infectieuze als niet-infectieuze complicaties. We besteden speciale aandacht aan de functie van T-cellen en mogelijke behandelingen die specifiek gericht zijn op het remmen van deze cellen. Dit onderzoek kan bijdragen aan een snellere diagnose en betere behandeling van primaire antilichaamstoornissen en de bijbehorende complicaties.

Deel 1: Diagnostiek en ziektemechanismen van primaire antilichaamstoornissen

In deel 1 van deze thesis doen we onderzoek naar genen die mogelijk betrokken zijn bij primaire antilichaamstoornissen. Door nieuwe genen te ontdekken die primaire antilichaamstoornissen kunnen veroorzaken, willen we bereiken dat meer patiënten de juiste antilichaamstoornis gediagnosticeerd krijgen. Daarnaast onderzoeken we de relatie tussen de afweercellen en erfelijke en niet-erfelijke antilichaamstoornissen, zodat we beter begrijpen welke afweercellen een belangrijke rol spelen bij verschillende antilichaamstoornissen.

In hoofdstuk twee beschrijven we een nieuwe genetische verandering in het RAC2-gen, genaamd RAC2-E62K, die onder andere primaire antilichaamstoornissen kan veroorzaken. We beschrijven drie patiënten met deze genetische verandering,

1 Granulomateuze lymfocytaire interstitiele longziekte

die last hadden van herhaalde luchtweginfecties en longproblemen. Met behulp van experimenten met afweercellen van deze patiënten laten we zien dat de verandering in RAC2 de functie van bepaalde afweercellen verstoort, waardoor deze cellen minder effectief zijn in het bestrijden van infecties.

In hoofdstuk drie beschrijven we hoe veranderingen in het MYB-gen mogelijk primaire antilichaamstoornissen kunnen veroorzaken. We beschrijven twee patiënten met een afweerstoornis in zowel B-cellen als T-cellen, en ernstige verstoorde bloedaanmaak. Met behulp van experimenten met afweercellen van een van deze patiënten laten we zien dat de verandering in MYB-gen mogelijk leidt tot een verstoorde ontwikkeling van T-cellen.

In hoofdstuk vier tonen we aan dat T-cellen in het bloed mogelijk een belangrijke rol spelen bij erfelijke vormen van primaire antilichaamstoornissen. Door experimenten met afweercellen van patiënten hebben we ontdekt dat verschillende erfelijke oorzaken kunnen worden onderscheiden door te kijken naar verschillende soorten T-cellen in het bloed. Echter zagen we ook dat bij deze patiënten juist minder T-cellen aanwezig waren in ontstoken darmweefsel, aangedaan door enteropathie. Dit suggereert dat T-cellen misschien niet zo belangrijk zijn bij enteropathie in patiënten met een erfelijke vorm van primaire antilichaamstoornissen als we dachten.

In hoofdstuk vijf leggen we uit dat het remmen van de groei van T-cellen moeilijker is bij patiënten met antilichaamstoornissen in vergelijking met gezonde mensen. Om dit te onderzoeken, hebben we afweercellen van patiënten in het laboratorium laten groeien met verschillende soorten medicijnen die de afweer remmen. Daarnaast hebben we gekeken of bij patiënten die behandeld werden met deze medicijnen hun niet-infectieuze complicaties verminderden. We hebben gezien dat als een patiënt goed reageerde op een medicijn, het medicijn ook de groei van T-cellen goed kon remmen in het lab.

Deel 2: Monitoring en behandeling van infectieuze en niet-infectieuze complicaties

In deel 2 van deze thesis doen we onderzoek naar de monitoring en behandeling van infectieuze en niet-infectieuze complicaties. Hiervoor gebruiken we verschillende informatie van verschillende grote klinische studies. Met deze studies willen we bruikbare risicofactoren ontdekken waarmee in de toekomst kan worden bepaald welke patiënten een groot risico hebben op infectieuze en niet-

infectieuze complicaties. Daarnaast vergelijken we met deze studies verschillende behandelingen voor infectieuze en niet-infectieuze complicaties met elkaar, zodat we kunnen bepalen welke behandelingen heet meest effectief zijn voor patiënten met antilichaamstoornissen.

In hoofdstuk zes vergelijken we de effectiviteit van antibiotica en van antilichamen van gezonde donoren bij patiënten met milde primaire antilichaamstoornissen. We hebben ontdekt dat beide behandelingen over het algemeen even effectief zijn, maar profylactische antibiotica hebben minder bijwerkingen, zijn gemakkelijker te gebruiken en goedkoper. Op basis van deze resultaten raden we profylactische antibiotica dus aan als eerste keuze om infecties te voorkomen bij milde primaire antilichaamstoornissen. Sommige patiënten reageerden echter niet goed op profylactische antibiotica en hadden nog steeds last van infecties. Voor hen kan behandeling met antilichamen van donoren wel effectief zijn om het aantal infecties te verminderen.

In hoofdstuk zeven onderzoeken we risicofactoren voor longschade door terugkerende infecties bij patiënten met primaire antilichaamstoornissen. We hebben ontdekt dat er gedurende het 10 jaar durende onderzoek weinig longschade ontstaat bij de meeste patiënten. Patiënten met de antilichaamstoornis XLA[2], patiënten met terugkerende infecties en patiënten die al longschade hadden, hebben een verhoogd risico op verdere schade. Het is dus belangrijk om deze patiënten nauwlettend te volgen. Profylactische antibiotica werden vaker gegeven aan patiënten met toenemende longschade, maar konden de toenemende longschade niet stoppen.

Hoofdstuk acht gaat over klinische kenmerken en symptomen waarmee GLILD vaak gepaard gaat en risicofactoren voor een slechte prognose. GLILD komt vaker voor bij vrouwen en gaat gepaard met een vergrote milt, vergrote lymfklieren, bloedarmoede, leverziekte, darmziekten en veranderingen in B- en T-cellen. Onbehandelde GLILD-patiënten hebben vaak verslechtering van de longfunctie en andere complicaties. Patiënten met veel GLILD-activiteit op CT-scans, leverziekte of veel NK-cellen hebben vaak een slechte prognose. Deze markers kunnen in de toekomst worden gebruikt om patiënten met een slechte prognose te identificeren en vroegtijdig te behandelen.In hoofdstuk negen beschrijven we de bestaande literatuur over de effectiviteit van gangbare behandelingen voor GLILD en doen

2 X-linked aggamaglobulinemie

we aanbevelingen voor specifieke behandelingen. GLILD is een niet-infectieuze complicatie waarbij de afweercellen het longweefsel aanvallen. Dit veroorzaakt ernstige ziekte en sterfte bij patiënten met primaire antilichaamstoornissen. Er is echter weinig onderzoek gedaan naar de beste behandelingsopties. Uit bestaande literatuur blijkt dat het medicijn rituximab, een middel wat specifiek B cellen opruimt, en combinatietherapie met rituximab mogelijk zeer effectief zijn als behandeling voor GLILD, terwijl corticosteroïden mogelijk minder effectief zijn. De meeste studies meldden dat corticosteroïden slechts bij een klein deel van de patiënten effectief waren. Dit kan te wijten zijn aan een vertekening van de bestaande literatuur, waarbij positieve resultaten voor lief worden genomen en daardoor niet worden gepubliceerd.

Daarom onderzoeken we in hoofdstuk 10 de effectiviteit en veiligheid van corticosteroïden als eerste keuze behandeling voor GLILD. Het blijkt dat corticosteroïden bij 42% van de patiënten leiden tot langdurige vermindering van GLILD. Bijwerkingen en infecties waren voornamelijk geassocieerd met langdurig gebruik van corticosteroïden tijdens onderhoudstherapie. Korte behandelingen zijn net zo effectief als langdurige behandelingen en geven weinig bijwerkingen. Daarom stellen we corticosteroïden als eerste keus behandeling voor GLILD, ondanks dat rituximab mogelijk effectiever is. We doen deze aanbeveling omdat de mogelijke bijwerkingen van corticosteroïden het beste beschreven zijn en corticosteroïden relatief weinig ernstige bijwerkingen hebben in patiënten met primaire antilichaamstoornissen.

In hoofdstuk 11 kijken we naar hoe we HLH[3] kunnen vaststellen. HLH is een syndroom waarbij het afweersysteem te actief is. Dit kan leiden tot ernstige orgaanschade en zelfs de dood. Daarom is het belangrijk om HLH snel te ontdekken en op tijd te behandelen om ernstige complicaties te voorkomen. We hebben onderzocht welke criteria ons kunnen helpen om HLH vast te stellen en ontdekt dat een paar belangrijke criteria samen gebruikt kunnen worden om HLH met veel zekerheid vast te stellen. De tests die nodig zijn voor deze criteria kunnen allemaal binnen 24 uur worden gedaan. Met deze aanpak kunnen we in de toekomst mogelijk de situatie van HLH-patiënten verbeteren door HLH sneller te ontdekken en te behandelen.

3 Hemofagocytaire lymfohistiocytose

Klinische aanbevelingen

Op basis van deze eerdere hoofdstukken kunnen we enkele belangrijke klinische aanbevelingen doen om de diagnose, monitoring en behandeling van primaire antilichaamstoornissen te verbeteren. Ten eerste is het belangrijk om te proberen een genetische diagnose te stellen bij patiënten met behulp van geavanceerde genetische technieken. Een genetische diagnose kan een grote impact hebben op de behandeling van een patiënt. In de toekomst kan er ook een internationaal platform worden ontwikkeld dat nieuwe genetische varianten meldt aan clinici en onderzoekers. Dit zou genetisch onderzoek vereenvoudigen.

Ten tweede kunnen we aangepaste versies van de celkweekproeven implementeren om de optimale behandeling voor elke individuele patiënt te selecteren. Primaire antilichaamstoornissen zijn verschillend bij elke patiënt, dus een gepersonaliseerde aanpak is vaak nodig. Door te kijken naar de samenstelling van afweercellen en de eiwitten die ze afscheiden, kunnen we de juiste medicatie selecteren.

Ten derde moeten patiënten met een hoog risico op infecties intensiever worden gemonitord. Ze kunnen profylactische antibiotica en/of een hogere dosis donor antilichamen krijgen om de infectielast te verminderen en het risico op longschade te verminderen. Patiënten met de antilichaamstoornis XLA lopen een hoog risico op ernstige luchtwegaandoeningen. Daarom zouden we voor deze patiënten ook onderzoek moeten doen naar stamceltransplantatie en gentherapie als behandelingsopties.

Tot slot kunnen we gestandaardiseerde behandelingen voor GLILD introduceren om de ziektelast en sterfte bij patiënten met antilichaamstoornissen te verminderen. Het is belangrijk om patiënten met een hoog risico op een slechte prognose vroegtijdig te herkennen en te behandelen. Meer gerandomiseerd klinisch onderzoek is echter nodig om de optimale behandeling voor GLILD verder te bepalen.

Conclusie en toekomstig onderzoek

In deze thesis beschrijven we meerdere onderzoeken die de zorg voor patiënten met antilichaamstoornissen sterk kunnen verbeteren. We weten nu beter welke patiënten we in de toekomst moeten screenen voor longschade. Daarnaast hebben we nieuw wetenschappelijk bewijs gevonden en bestaand bewijs samengevat voor

de behandeling van GLILD. Met dit bewijs kan er een eerste behandelrichtlijn voor GLILD geschreven worden.

Toekomstig onderzoek moet zich richten op internationale samenwerking, verbetering van celkweekproeven, ontwikkeling van nieuwe behandelingen om de infectielast te verminderen, en uitvoeren van gerandomiseerde klinische onderzoeken om behandelingsopties voor GLILD te vergelijken. Het doel is om de diagnose, behandeling en monitoring van afweerstoornissen verder te verbeteren en gepersonaliseerde zorg te bieden aan patiënten die niet reageren op standaardbehandelingen.

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ACKNOWLEDGEMENTS

"Many ideas grow better when transplanted into another mind than the one that they sprung up." – O.W. Holmes

Veel van de projecten in dit proefschrift zijn al jaren geleden uitgedacht en gestart door anderen. Dit proefschrift is dan ook een team effort en had nooit tot stand kunnen komen zonder de inzet van velen.

Allereerst wil ik alle deelnemers ontzettend bedanken. Zonder jullie inzet was dit proefschrift nooit ontstaan. Heel erg bedankt dat jullie in grote getalen langs zijn gekomen voor alle studie visites en extra bloedafnames en dat jullie al onze vragenlijsten hebben ingevuld. Bedankt ook voor het bezoeken van de patiënten dagen en voor jullie input. De vragen die jullie ons stelden, hebben geleid tot een aantal van de onderzoeken in dit proefschrift en ik hoop dan ook dat de uitkomsten hiervan jullie vragen voor een deel beantwoorden.

Leden van de promotiecommissie prof. dr. F. van Wijk, prof. dr. L.J. Bont, prof. T.W. Kuijpers, prof. dr. D. Hamann, prof. dr. J. Frenkel, dr. A. Rutgers, hartelijk dank voor het beoordelen van dit proefschrift. Dear prof. dr. K. Warnatz, dear Klaus, thank you very much for your critical review of this thesis. I am really honored to defend my thesis in your presence. It was a true joy to collaborate with you so closely the past few years and be allowed to work with you on the STILPAD project. Your enthusiasm, empathy and feedback were a true inspiration for me as a young investigator and made our countless meetings over STILPAD really pleasant. Thank you for giving me the opportunity to work with you.

Veel dank aan mijn promotieteam voor al hun vertrouwen en steun: promotor prof. dr. N.M. Wulffraat en dr. J. van Montfrans en copromotoren dr. S. Nierkens en dr. M. Boes

Nico, heel erg bedankt voor de adviezen en feedback die ik van je heb mogen ontvangen tijdens onze gesprekken. Je hebt me een aantal keer enorm geholpen om de zaken van verschillende kanten te bekijken en ook te durven kijken naar waar mijn eigen belangen en ontwikkelmogelijkheden liggen.

Joris, de afgelopen jaren ben jij voor mij een rots in de branding geweest. Ik wil je bedanken voor het enorme vertrouwen wat je vanaf het begin af aan al in

mij gehad hebt. De autonomie die ik vanaf dag 1 al in de samenwerking met jou ervaarde was echt heel prettig en hiermee heb je me in mijn kracht weten te zetten. Tegelijkertijd was je er altijd om even over mijn schouder mee te kijken, advies te vragen, coachen en bijsturen waar nodig. Als ik in de put zat omdat iets niet lukte, was je er altijd voor een kop koffie, een luisterend oor en een oppeppende speech. Tegelijkertijd was je er ook altijd om de dingen die wel lukte, groot of klein, te vieren. Hierdoor heb ik me kunnen ontwikkelen tot de zelfstandig wetenschapper die ik nu ben en ook daarvoor wil ik je heel erg bedanken. Als anderen aan mij vragen waarom ik het zo naar mijn zin had op mijn werk, vertelde ik vaak over jou en hoe prettig de samenwerking was tussen ons. Ik ga onze vele kopjes koffie en gesprekken dan ook echt missen, maar hoop dat we ook in de toekomst nog af en toe samen kunnen blijven werken.

Stefan, zonder jou was ik überhaupt nooit aan dit boekje begonnen en ik wil je daarom ook heel erg bedanken. Het is inmiddels alweer ruim zeven jaar geleden dat ik bij jou het kantoor instapte voor een gesprek over mijn masterscriptie met JJ en jou. Op dat moment was ik niet eens van plan om verder ooit nog iets met wetenschap te doen, maar onze samenwerking heeft dat permanent veranderd. Het translationele onderzoek in jullie groep wakkerde bij mij weer een enthousiasme voor de wetenschap aan. Jouw optimistische en opgeruimde manier van leidinggeven maakt het onwijs verfrissend om met je samen te werken. Met je tomeloze stroom van ideeën wist je mij altijd te inspireren op de momenten dat ik vastliep. Bedankt voor alle tijd die je in mij hebt gestoken om mij van feedback en inzichten te voorzien en mijn krakkemikkige eerste pogingen tot een research paper na te kijken. Bedankt dat je mij hebt doen inzien dat onderzoek doen een team effort is, dat samenwerken je sterker maakt en je bewust doet worden van dingen waar je onbewust onbekwaam in bent.

Marianne, door jouw onconventionele kijk heb je de inhoud van dit boekje, mijn PhD traject en mijn kijk op de wetenschap blijvend veranderd en daarvoor wil ik je dan ook enorm bedanken. Iedere keer als ik dacht te hebben uitgedokterd welke kant het op zou moeten gaan, kwam jij met een kritische vraag of onverwacht inzicht. Ondanks dat dit ook af en toe frustrerend kon zijn heeft dat me ook gestimuleerd om verder te kijken dan mijn eigen neus lang is. Samen met Stefan wist je me altijd uit te dagen om naar de biologische mechanismen achter mijn hypotheses en resultaten te blijven zoeken en dus buiten mijn eigen comfort zone te treden. Hierdoor heb je mijn blik enorm verbreed en mijn immunologische kennis verdiept, waarvoor dank.

Helen, zonder jouw enorme energie en kritisch vragenvuur waren de projecten in dit boekje nooit tot wasdom gekomen. Bovendien, zonder al het voorwerk wat jij samen met Roos, Stefan, Joris, Marianne en Femke de afgelopen jaren gedaan hebt, was dit boekje er nooit geweest en daarom wil ik je ook bedanken. Ik heb met heel veel plezier met je samengewerkt en vind het enorm inspirerend om te zien hoe kennis van klinisch en fundamenteel onderzoek gebundeld kan worden om zo patiënten met zeldzame ziektes een behandeling aan te bieden. Ik hoop dat ik later ook op zo een manier arts kan zijn. Daarnaast wil ik je ook bedanken voor al die keren dat je me op sleeptouw hebt genomen op de congressen die we samen hebben bijgewoond. Het was altijd enorm gezellig om met jou te borrelen en bovendien leidde zo'n borrel vaak ook nog eens tot een kans om de experts binnen ons onderzoeksveld persoonlijk te leren kennen.

I would also like to thank all the other co-authors and collaborators that were involved in any of the projects I worked on during my PhD. I would love to specifically mention Sigune Goldacker; it is a real pleasure to work with you. You're structured way of working, critical questions regarding methodology and keen eyes for detail are really complementary to my own pitfalls and without you I could have never unraveled the complex database of the STILPAD study. Ilona bedankt voor de begeleiding die je mij hebt gegeven bij het schrijven van mijn allereerste clinical trial research paper. Door jouw opbouwende kritiek heb ik mijn schrijfvaardigheid enorm kunnen verbeteren. Virgil, Hanna en Astrid, ik wil jullie enorm bedanken voor de prettige samenwerking tussen Rotterdam en Utrecht. Ik ben er echt van overtuigd dat deze samenwerking het PID-onderzoek in Nederland verder helpt en ik ben dan ook erg blij dat deze samenwerking ook na mijn PhD wordt voortgezet.

Ik wil ook mijn mede PID-PhD'ers Roos, Mischa, Marianne, Stefanie, Sharisa en Amir bedanken. Het was onwijs gezellig en leerzaam om samen met jullie te vergaderen, te borrelen, naar congressen te gaan en papers te schrijven. Het was heel erg fijn om met jullie allemaal samen te werken en om met jullie de sores van het PhD leven te kunnen delen. Zelfs monitor visitaties voorbereiden en tot in de late uurtjes pipeteren werd dragelijk dankzij jullie!

I'd also like to thank the entire BO-NI-LI group. Thank you for letting me be part of your group! It was really nice to have the feeling to be part of something. Even though I was quite adrift sometimes during my PhD, you guys would always make me feel like I belonged in the CTI and in your group. I'd specifically like to thank Celina and Coco; you guys were the first to welcome me to the group and you did a

great job. Moreover, I'd like to thank Ester, Annelisa and Vania. Thank you for all the time you guys spent explaining me lab techniques, Fortessa settings and antibody stainings! Ester I am really going to miss another rabbit enthusiast in my life. It was really a joy to work with you! Annelisa and Vania, after working in mostly empty offices you guys welcomed me into the Sunshine Room with open arms. It really changed my PhD for the better. Thank you both for all the small talk, cortados, cookies, a shoulder to cry on and critical scientific discussions, but please do not offer me any wodka soaked gummy bears ever again!

Ik wil ook de rest van de Sunshine room bedanken. Eveline, Anniek, Mara, Mitchell, bedankt voor alle gezelligheid en fijne gesprekken, voor enorm gezellige vrijdagmiddagborrels die verschrikkelijk uit de hand zijn gelopen. Anniek, jij in het bijzonder heel veel dank voor het nakijken van een deel van mijn thesis! Mara, jij bedankt dat je me meenam naar de Kuball borrels, dat we nog vele salmari (en andere rare shotjes) mogen drinken samen!

Lieve Roos en lieve Marleen, wat superfijn dat jullie mijn paranimfen willen zijn. Ik vind het echt een eer om door jullie twee vergezeld te worden op deze prachtige dag. Het was heel bijzonder om voor jullie allebei paranimf te zijn en vind het heel speciaal om jullie er allebei bij te hebben. Roos, zonder jou was dit boekje er niet gekomen. Jij hebt me samen met Stefan bij Joris voorgedragen als PhD-kandidaat en was, zeker in het begin, nauw betrokken bij het opzetten van mijn projecten. Dankzij jouw creatieve ideeën en goede raad heb ik echt een onwijs goeie start gehad en daar wil ik je voor bedanken. Bovendien wist jij me vaak uit te dagen om net een stapje extra te zetten, wat echt onwijs motiverend was. Tot slot, was het ook heel fijn om samen stoom af te kunnen blazen op borrels, fuifjes en verkleedfeestjes om zo alle sores van het SUMMA en het PhD leven even te kunnen vergeten.
Marleen ook jij hebt mij enorm ondersteund tijdens het maken van dit boekje. Direct, door mijn thuis-kantoorgenootje te zijn tijdens de coronapandemie. Ik vond het echt heerlijk hoe we samen konden discussiëren over wetenschap en statistiek en elkaar konden helpen als we er even niet uitkwamen en samen de dag afsloten met een wijntje op het balkon. Maar ook indirect, door er al bijna 20 jaar voor me te zijn met soms een arm om me heen en soms een schop onder mijn kont. Er zijn een boel heftige dingen gebeurd de afgelopen 5 jaar en daarin heb je mij en Amber heel erg bij kunnen staan. Ik heb dan ook echt het gevoel dat onze vriendschap de afgelopen jaren nog verder verdiept is en dat vind ik heel fijn en bijzonder. Bedankt voor alles en bedankt voor dat je bent wie je bent.

Lieve Boris en lieve Raisa, jullie wil ik natuurlijk ook bedanken. Jullie hebben allebei een bijzondere plek in mijn hart. Jullie zijn twee energieke wervelwindjes die altijd voor reuring zorgen en geven mijn leven kleur. Bedankt voor alle biertjes, wijntjes, kletspraatjes, serieuze gesprekken, vakanties, festivals, feestjes, knuffels en kusjes, dat er nog veel mogen volgen. Ik vind het mooi om te zien hoe we deze nieuwe fase van onze vriendschap, met kinderen, weer op een nieuwe manier shine geven en we op zoek zijn naar nieuwe tradities. Ik hoop dat dineren bij jullie op het dakterras, met Marleen, er daar zeker een van blijft.

Lieve Floris, een dankwoord voor jou kan natuurlijk niet uitblijven, want ook jij hebt mijn leven enorm kleur gegeven en ik mis je verschrikkelijk. Ik weet zeker dat ik je op deze bijzondere dag nog een beetje extra ga missen. Ik mis samen voetbal kijken, samen spelletjes doen, samen bier drinken en serieus (of minder serieus) praten over het leven. Ook tijdens mijn PhD deden we dat vaak en dan kon het jou geen klap schelen als ik om 21.00 ’s avonds, 2 uur later dan afgesproken, uit het lab kwam rollen en nog niet gegeten had. Dan regelde je wat te eten en een biertje en maakte we er alsnog een fijne avond van. Ik mis je nuchtere blik, ik mis je relativeringsvermogen en ik mis je knuffels, maar ben ook heel blij dat je een onderdeel hebt mogen zijn van dit alles. Bedankt dat je in mijn leven was lieverd.

What’s up nerds? Daan, Robert, Max, Ilia, Thijs (en Boris en Floris ook) ik wil jullie allemaal ook heel erg bedanken. De vele D&D sessies van de afgelopen jaren waren een ontzettend fijne afleiding van het PhD leven en de weekendjes weg die erbij hoorde koester ik als ontzettend fijne herinneringen. Daan en Ilia, jullie wil ik als co-DM in het bijzonder bedanken. Ik vind het enorm tof om met jullie allebei de meest bizarre avonturen te hebben bedacht en nog steeds te bedenken. Ik hoop dat we in de toekomst nog veel verzonnen avonturen met elkaar mogen beleven.

Lieve andere bear buddies, Froukje, Arian, Lotta, Simone, Jelmer, Anne-Fleur, Bas, Gina, Dagmar, Jeroen, Derk, Isi, Jochum, Joris, Nikki en Norma heel erg bedankt voor alle feestjes, festivals, barbecues en park-chillings. Ik hou van jullie en het is altijd weer een genot om met jullie samen te zijn!

Lieve SUMMies, Jonas, Wimkees, Sophie en Jeroen, Tanja en Leo, Fieke en Luc en Jonathan en Emma. Bedankt voor alle borrels, het fantastische SUMMA weekend, (verkleed)feestjes, “Poesjes” kijken (voor de lezer die dit programma niet kent, ga het kijken via NPO start), band repetities en spelletjes avonden. Bedankt voor de

band de we tijdens de studie hebben opgebouwd en bedankt voor alle lol die we samen hebben gehad.

Lieve Wombats, Marnix, Hans, Mike, Fons, CJ en Kelly. Bedankt dat ik de afgelopen jaren met jullie op het veld heb mogen staan. Ik zeg mogen, want het getuigd van enorme mentale weerbaarheid om mijn geklungel elke zondag maar weer toe te laten. Gelukkig is gezelligheid in ons team de belangrijkste factor, want dat is iets waar ik dan wel weer in uitblink en ondanks dat mijn honkbal carrière er voorlopig op zit hoop ik jullie snel weer te zien voor een borrel of een etentje.

Lieve Hedzer en lieve Loes, ontzettend bedankt voor jullie steun de afgelopen jaren. Ik vind het heel bijzonder hoe jullie deur altijd voor ons open staat en hoe jullie mij het gevoel weten te geven dat ik erbij hoor. Bedankt voor alle gezellige etentjes, uitjes en vakanties. Hedzer, ik heb het heel erg prettig gevonden om met jou als mede wetenschapper over mijn onderzoek te kunnen praten. Ik vind het zo tof om te zien hoeveel interesse je voor mijn onderzoek hebt en ben dan ook heel blij dat ik je door het laboratorium heb kunnen rondleiden. Loes, bedankt dat je er altijd voor weet te zorgen dat iedereen zich binnen het gezin gezien voelt en bedankt voor al je verdiepende vragen. Ik vind het heel bijzonder hoe je in onze gesprekken mijn blik naar het interpersoonlijke vlak weet te trekken, ook al vind ik het niet altijd makkelijk om je vragen daarover te beantwoorden, bedankt daarvoor.

Lieve Lykele en lieve Minou, bedankt voor alle enorm lieve gebaren, woorden en daden van de afgelopen jaren. Jullie zijn allebei enorm lieve, zorgzame, attente en empathische mensen en ik vind het dan ook enorm fijn dat we zo dicht bij elkaar staan. Bedankt voor alle lunches, borrels, etentjes, spelletjes avonden en vakanties en bedankt dat jullie ons zo betrekken bij jullie gezin. Ik vind het echt heel bijzonder om Fjorre en Teije van zo dichtbij op te zien groeien en een heel fijn idee dat jullie Marre ook van zo dichtbij mee gaan maken.

Lieve papa en mama, jullie verdienen een prominent plekje in dit dankwoord. Het is moeilijk om de juiste woorden te vinden om jullie te bedanken. Want welke woorden doen recht aan de liefde en dankbaarheid die een kind voelt voor zijn ouders? Jullie zijn mijn steun en toeverlaat en hebben mij zien vallen en opstaan de afgelopen jaren. Bedankt dat jullie me hebben kunnen loslaten, zodat ik zelf kon voelen hoe het is om te vallen. Bedankt dat jullie er ook altijd zijn om me weer te helpen met opstaan. Het moet niet makkelijk zijn om je kind fouten te zien maken, waarvoor je ze liever zou behoeden. Bedankt voor de normen en waarden die

jullie aan mij mee hebben gegeven en dat jullie mij er gaandeweg aan hebben herinnerd hoe belangrijk deze normen en waarden zijn. Jullie leerden me dat ergens goed in zijn 10% talent is en 90% discipline en deze les heb ik meerdere malen tegen mezelf moeten herhalen tijdens het schrijven van dit boekje en is voor mij een soort mantra als het leven tegen zit. Dankzij jullie heb ik geleerd dat een afspraak een afspraak is, een les die cruciaal is bij het bedrijven van wetenschap. Jullie leerden me ook dat je veel levensgeluk haalt uit het benutten van je talenten. Lieve papa en mama, ik wil jullie bedanken voor deze, en voor alle andere levenslessen die jullie mij hebben geleerd. Zonder jullie als gids, was dit boekje er niet alleen nooit geweest, maar was ik ook niet de persoon geweest die ik nu ben. Bedankt en ik hou van jullie!

Lieve Maaike, Jelte en Fedde, bedankt voor alle steun en hulp die Amber en ik de afgelopen jaren van jullie hebben mogen ontvangen. Maaike, bedankt dat je altijd voor me klaarstaat om even te sparren, een sollicitatiebrief na te kijken of om gewoon gezellig te kletsen. Je bent een onwijs fijne grote zus en ik kijk heel erg uit naar de nieuwe fase die we in gaan nu we allebei kinderen hebben. Jelte bedankt voor alle hulp met het klussen hier in huis en al het gereedschap dat Amber en ik hebben mogen lenen. Zonder al jouw hulp en expertise waren we nu nog steeds bezig geweest met stuccen, kitten en plinten plakken. Bedankt dat je er bent voor mijn zus en voor Fedde. Fedde, ik wil jou ook bedanken. Met jouw blije energie weet je me altijd op te vrolijken. Door samen te spelen en te voetballen, samen praatjes te maken en samen te stoeien weet je het altijd gezellig te maken. Ik vind het ook heel tof om te zien dat je zo'n trotse grote neef bent voor Marre en weet zeker dat je haar met jouw positieve energie ook heel blij gaat maken.

Lieve Erik bedankt dat je zo'n fijne grote broer bent. Bedankt voor alle etentjes, bezoekjes aan Ed, samen ijshockey kijken en spelletjes spelen. Bedankt ook dat je altijd eerlijk bent, ook als het moeilijk is om te horen. Door jou zijn we als familie weer dichter bij elkaar gekomen. Dat vind ik echt heel erg fijn en ik ben je daar dan ook onwijs dankbaar voor.

Lieve Amber en Marre ik wil dit dankwoord graag afsluiten door jullie te bedanken. Amber, je bent mijn maatje al bijna een half leven lang en je steunt me door dik en dun. Je bent onwijs lief, zorgzaam en meegaand, een doorzetter en een harde werker, zacht als het kan en stevig als het moet. Doordat we elkaar al zo lang kennen, zijn we echt samen volwassen geworden en ik vind het heel mooi om te zien hoe jij tot bloei bent gekomen in die periode. Ons leven samen heeft al veel

pieken en dalen gekend en ik ben trots hoe we hier ons samen door heen slaan. De afgelopen jaren heb je me heel veel ruimte gegeven, niet alleen om tot laat in het lab te staan of om in de vakantie nog even aan die ene paper te werken, maar ook om mijn eigen hobby's en interesses te achtervolgen en tijd voor mijzelf te nemen. Ik weet dat dit niet altijd makkelijk was en vind het daarom extra bijzonder dat je dit toch bent blijven doen. Lieverd, ik hou ontzettend veel van je en ben helemaal gek op je, bedankt dat je bent wie je bent en bedankt dat je er altijd voor mij bent. Marre, je bent er pas kort, maar inmiddels kan ik me niet meer voorstellen dat je er ooit niet bent geweest. Ik was zo blij toen Amber me vertelde dat ze zwanger was van jou en ook al zat ik aan de andere kant van de wereld, toch voelde ik me gelijk met je verbonden. In verwachting van jou heb ik de hand gelegd aan het grootste deel van dit boekje en het vooruitzicht van jouw komst gaf me de positieve energie die ik nodig had om dit boekje af te maken. Heel erg bedankt daarvoor lieverd, ik hou heel veel van jou en ben onwijs blij dat ik nog heel lang van je kan genieten.

ABOUT THE AUTHOR

Bastiaan Marcus Smits was born on the 17th of January 1990 in Utrecht. He completed his secondary school in 2010. That year he started his biomedical sciences study in Amsterdam. During his study he focused on immunology, oncology and human physiology research, but also followed interdisciplinary honours courses on financial geography, environmental sciences, neuropsychology and quantum physics to broaden his horizon. In his final year he worked as a research intern at the Department of Oncogenomics in the group of Prof. Rogier Versteeg, where he investigated the role of the BMP signaling pathway in neuroblastoma.

His education inspired him to apply for the selective Utrecht medical master (SUMMA) program at the University of Utrecht. During his medical training he took an interest to internal medicine and did additional internships in pulmonology, intensive care medicine and neurology. During the SUMMA program he came into contact with Dr. Stefan Nierkens and Dr. Jaap Jan Boelens, who offered him an intern position at the Center for Translational Immunology at the University Medical Center Utrecht. During this internship he set-up a national retrospective cohort study that investigated diagnosis and treatment of HLH. The results of this cohort study are partially published in this thesis.

After he finished his medical training in 2018 Dr. Stefan Nierkens recommended him to apply for a PhD position with Dr Joris. van Montfrans at the department of Pediatric Immunology and Infectious Disease, with which Dr. Nierkens had a collaboration. During this PhD he collaborated closely with the group of Dr. Marianne Boes and with the group of Dr. Stefan Nierkens at the Center for Translational Immunology and with the group of Prof. Klaus Warnatz at the Department of Immunodeficiency at Freiburg University.

Bas lives in Utrecht together with Amber and their daughter Marre. He is currently working as resident-not in training in internal medicine at the St. Antonius Hospital in Nieuwegein.