COMMON VARIABLE IMMUNODEFICIENCY

to solve the variable of the equation

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COMMON VARIABLE IMMUNODEFICIENCY
to solve the variable of the equation

COMMON VARIABLE IMMUNODEFICIENCY
de variabele in de vergelijking oplossen

(met een samenvatting in het Nederlands)

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-You can't start a fire without a spark-
Bruce Springsteen, *Dancing in the Dark*

CONTENTS

7

GENERAL INTRODUCTION

GENERAL INTRODUCTION

"Patients with primary immunodeficiencies:
the greatest teachers of modern immunology"
Robert A. Good, pediatrician and scientist (1922- 2003)

Primary immunodeficiencies (PIDs) are inherited defects of the immune system, having diverse clinical characteristics but an increased susceptibility to various types of infections as a common denominator. Despite their relatively low prevalence, PIDs are important diseases to study. First and most importantly, they are severe disorders with high morbidity and mortality; for example, severe combined immunodeficiency (SCID) is lethal within the first year of life unless treated with hematopoietic stem cell transplantation (HSCT). Second, they "…are 'experiments of nature' which, properly considered, may permit acquisition of new and useful knowledge applicable far beyond the patients and diseases studied".[1] Currently, over 170 PIDs have been described and categorized based on the origin of the prominent defect: combined immunodeficiencies (T and B cell defects), predominantly antibody deficiencies (B cell focused), other well-defined immunodeficiency syndromes (including DNA repair defects in leukocytes), and diseases of immune dysregulation (mainly natural killer and cytotoxic T cell-related). Additional PIDs occur as consequence of congenital defects causing decreased phagocyte number, function, or both, and defects in innate immunity, including autoinflammation and complement deficiencies.[2] For these 170 entities, genetic causes have been unraveled; most are monogenetic diseases that follow a Mendelian inheritance pattern and present early in life. Conversely, PIDs that present after infancy have often polygenic origins and hence are complex to untangle. With an estimated overall prevalence of 1 in 10,000 live births, severe PIDs are uncommon.[3] Antibody deficiencies are the most numerical, especially in the adult population, encompassing half of all PID patients.[4]

Features of common variable immunodeficiency

The antibody deficiency referred to as common variable immunodeficiency (CVID) is the most common symptomatic PID, with an estimated prevalence of 1 in 25,000-50,000 Caucasians. In European PID registry databases, CVID accounts for 14[5] to 20%[4] of registered PID patients. It is a tremendously heterogeneous disease, characterized by recurrent infections of predominantly the respiratory tract; often, disease-related complications such a as severe autoimmunity develop. A diagnosis of CVID is made by identifying hypogammaglobulinemia of immunoglobulin (Ig) G in combination with IgA and/or IgM, recurrent bacterial infections and a poor specific antibody induction upon vaccination.[6] To allow for CVID diagnosis, direct or indirect causes of hypogammaglobulinemia need to be excluded first. The disease is generally not formally diagnosed until the age of 4, in order to rule out transient hypogammaglobulinemia of infancy. Nonetheless, a certain amount of overlap remains,

both clinical and immunological, between CVID and other antibody deficiencies with normal numbers of B cells, including specific antibody deficiency (SAD, with normal Ig concentrations), selective IgA deficiency, isolated IgG subclass deficiency and IgA with IgG subclass deficiency; often referred to as 'CVID-like diseases'.[2] CVID can manifest at any age; age of diagnosis follows a Gaussian distribution and the disease thus commonly presents between the 2nd and 4th decade of life.[7] About one-quarter of CVID patients is diagnosed in childhood, but literature on pediatric patients remains limited.

CVID patients are routinely treated with Ig replacement therapy, either intravenously (IVIG) or subcutaneously (SCIG), as the latter has several practical advantages. The introduction of IVIG over 30 years ago has had a significant positive impact on the morbidity and mortality of the disease, by reducing the frequency of severe and invasive infections. In most patients, the rate and severity of infectious events is reasonably well controlled by Ig replacement therapy and (prophylactic) antibiotics. Notwithstanding, some shortcomings remain. Firstly, IVIG is usually administered every 3-4 weeks over a time course of several hours, most often in the hospital setting, while SCIG is administered weekly, illustrating the time commitment required. Secondly, from the health care provider's perspective, the combined expenses of the Ig preparations and related hospital costs pose a high burden on the health care budget.[8;9] Thirdly, breakthrough infections still occur occasionally and subclinical infections and progression of disease sequelae can take place despite treatment. Fourthly, Ig replacement therapy does not prevent the occurrence of the major types of disease-related complications.

The foremost problem for the majority of CVID patients is the development of complications. These can be divided into disease consequences secondary to recurrent infections, such as bronchiectasis. Additionally, there are intrinsic CVID-related complications, including autoimmune diseases (typically autoimmune cytopenias, but also rheumatoid arthritis, systemic lupus erythematosus and organ-specific autoimmunity), lymphoproliferative disorders, granulomatous disease, enteropathy and malignancies.[10] Intrinsic complications affect two-third of the adult CVID population and are the major culprits of CVID morbidity and mortality, as the survival of afflicted patients is decreased.[7] CVID-related complications are habitually difficult to treat, requiring aggressive immunosuppressive regimens that are evidently of high risk in immunodeficient patients.[11;12]

CVID classifications

For the aforementioned reasons, it is of major relevance to monitor patients accurately and detect CVID-related complications as swiftly as possible; predictive risk factors could be supportive. This is especially important for children with CVID, as they on average have a longer life-span to develop sequelae. With the purpose to detect patients prone to develop complications, several CVID classifications have been made, dividing this variable disease into more homogeneous subclasses *(table 1)*. Classifications systems based on percentages of B cell[14;16;17;19] or T cell[18] subsets show that particularly patients with reduced proportions of Ig-class-switched memory B cells

are susceptible to intrinsic CVID-related complications, although these data have not been confirmed prospectively. We noted that all but one classifications were based on large cohorts of almost exclusively adult CVID patients, and argue that it remains to be investigated whether these are applicable to pediatric CVID patients as well, which was subject of study in this thesis.

Etiology and pathogenesis

Considering that the hallmark of CVID is hypogammaglobulinemia, it is plausible that many patients have a primary defect in the B cell lineage. Most CVID-related research is therefore focused on B lymphocyte development, differentiation, proliferation and homeostasis. In general, peripheral CVID B cells are normal in numbers, but skewed towards the naive phenotype; proportions of transitional B cells are increased,[20] while especially Ig-class-switched memory B cells are low.[21;22] Moreover, somatic hypermutation of the B cell receptor is often diminished.[15;23] *In vitro* studies clarified that there is often impaired Ig and cytokine production,[13;24] and defective upregulation of costimulatory molecules.[25;26] In a subset of patients however, the B cell defect is likely to result from non-B cell-intrinsic defects, e.g., defective T-cell help[27] or inadequate architectural structure of the lymph nodes. This is supported by the finding that besides B cell defects, numerous patients have abnormalities in T cell subsets,[18;24;28-49] natural killer (NK) cells,[50] invariant NK T cells,[51-53] dendritic cells (DCs),[54-58] monocytes [53;59-64] or innate signaling pathways (*figure 1*).[58;65]

This thesis is focused on primary B cell defects. Despite all efforts, a molecular diagnosis remains absent in the majority of patients. Some monogenetic defects have been found in sporadic CVID(-like) families, including defects in inducible costimulator (ICOS),[27] CD19,[66] CD20,[67] CD21,[68] CD27 (van Montfrans *et al.*, submitted) and CD81.[69] TNF receptor defects (TACI, BAFFR) were found in several patients,[70-72] but the general consensus is that heterozygous -and in some cases homozygous- mutations in TACI cause an increased susceptibility for disease and are not disease causing per se.[73-75] The currently known coding exons of candidate gene loci encoding cytokine/receptor pairs that were either involved in terminal B cell differentiation, preferentially expressed in germinal centers, or related to class switch recombination, have been sequenced in CVID families; however, this did not yield any disease-causing mutation.[76]

Taken together, the current data underscore that (intrinsic) B cell defects constitute the major underlying cause for CVID, but molecular evidence is not yet available for most of the CVID patients. Furthermore, there is a necessity to gather more information on diagnosis and treatment of the main clinical problems in CVID. The aim of this thesis is therefore two-fold:

I. To study B cell abnormalities in CVID, by investigating phenotypic and functional B cell defects
II. To address diagnostic and etiological aspects of CVID-related pulmonary (a) and gastrointestinal disease (b)

Table 1: Overview of the currently available classification methods for CVID.

Classification	Method	Subgroups
'London'	*In vitro* B cell culture with anti-IgM and IL-2	A) no Ig secretion B) IgM alone C) both IgM and IgG secreted -- one group lacking B cell
'Freiburg'	FACS	I) <0.4% $CD27^+IgM^-IgD^-$ B cells on lymphocytes a: >20% $CD21^-$ B cells b: <20% $CD21^-$ B cells II) >0.5% $CD27^+IgM^-IgD^-$ B cells on lymphocytes
'Paris'	FACS	MB0: <11% $CD27^+$ B cells on total B cells MB1: ≤8% $CD27^+IgD^-$ B cells on total $CD19^+$ B cells, with normal/increased subset of $CD27^+IgD^+$ B cells MB2: Near-normal subset distribution
EUROClass	FACS	B-: ≤1% B cells B+: > 1% B cells smB+: >2% switched memory B cells $smB+21^{norm}$: <10% $CD21^{low}$ B cells $smB+21^{lo}$: ≥10% $CD21^{low}$ B cells smB-: ≤2% switched memory B cells $smB\text{-}Tr^{norm}$: <9% CD38++IgM^{high} transitional B cells $smB\text{-}Tr^{hi}$: ≥10% CD38++IgM^{high} transitional B cells *OR:* $smB\text{-}21^{norm}$: <10% $CD21^{low}$ B cells $smB\text{-}21^{lo}$: ≥10% $CD21^{low}$ B cells
T cell-based	FACS	3 tertiles of naive $CD4^+CD45RA^+CD62L^+$ T cell proportions (%): low, normal, high I) <15% naive of total $CD4^+$ T cells II) 16-29% naive of total $CD4^+$ T cells III) 30%< naive of total $CD4^+$ T cells
Clinical phenotype-based	Clinics	1) No complications 2) Autoimmunity 3) Polyclonal lymphocytic infiltration 4) Enteropathy 5) Lymphoid malignancy
B cell phenotype-based in children	FACS	I) <5 $CD19^+CD27^+IgM^-$ B cells/uL II) ≥5 $CD19^+CD27^+IgM^-$ B cells/uL

FACS=fluorescence-activated cell sorting; Ab=antibody; ♂= male; ♀= female; RR=relative risk; ns=not specified

Association with clinics	Population	Ref
related to gender	--	[13]
Clustering autoimmune cytopenia and splenomegaly in Ia. Ko *et al.*: lower IgG , poorer pneumococcal Ab responses and more autoimmune and granulomatous disease in group I.	Ref 11: n=38 [≥18 y] (24 ♂, 14 ♀) Ref 12: n=53 [3-77 y] (19 ♂, 34 ♀)	[14] ; applied by [15]
MB0: higher prevalence of splenomegaly, lymphoid proliferation and granulomatous disease MB1: frequent splenomegaly	n=57 patients [22- 80 y] (20 ♂, 37 ♀)	[16]
smB-: more splenomegaly and granuloma smB-Tr^{hi}: more lymphadenopathy smB+21^{lo}: more splenomegaly and granuloma smB-21^{lo}: more splenomegaly and granuloma	n=303 patients [10-84 y] (133 ♂, 169 ♀)	[17]
Negative correlation between naive CD4^{+} T cells and overall clinical severity scores (r=-0.68) and levels of splenomegaly (r=-0.76) Associated with Freiburg (concordance 58.8%)	n=60 patients [15-78 y] (31 ♂, 29 ♀)	[18]
Decreased survival group 2-5 compared to group 1: 2) RR 2.5 3) RR 3.0 4) RR 4.0 5) RR 5.5	n=424 [11-90 y] (177 ♂, 128 ♀, 19 ns)	[7]
Only group I had meningitis, sepsis, bronchiectasis, granulomatous lung disease, autoimmune cytopenias, or hematologic malignancies	n=45 [2-19 y] (30 ♂, 15 ♀)	[19]

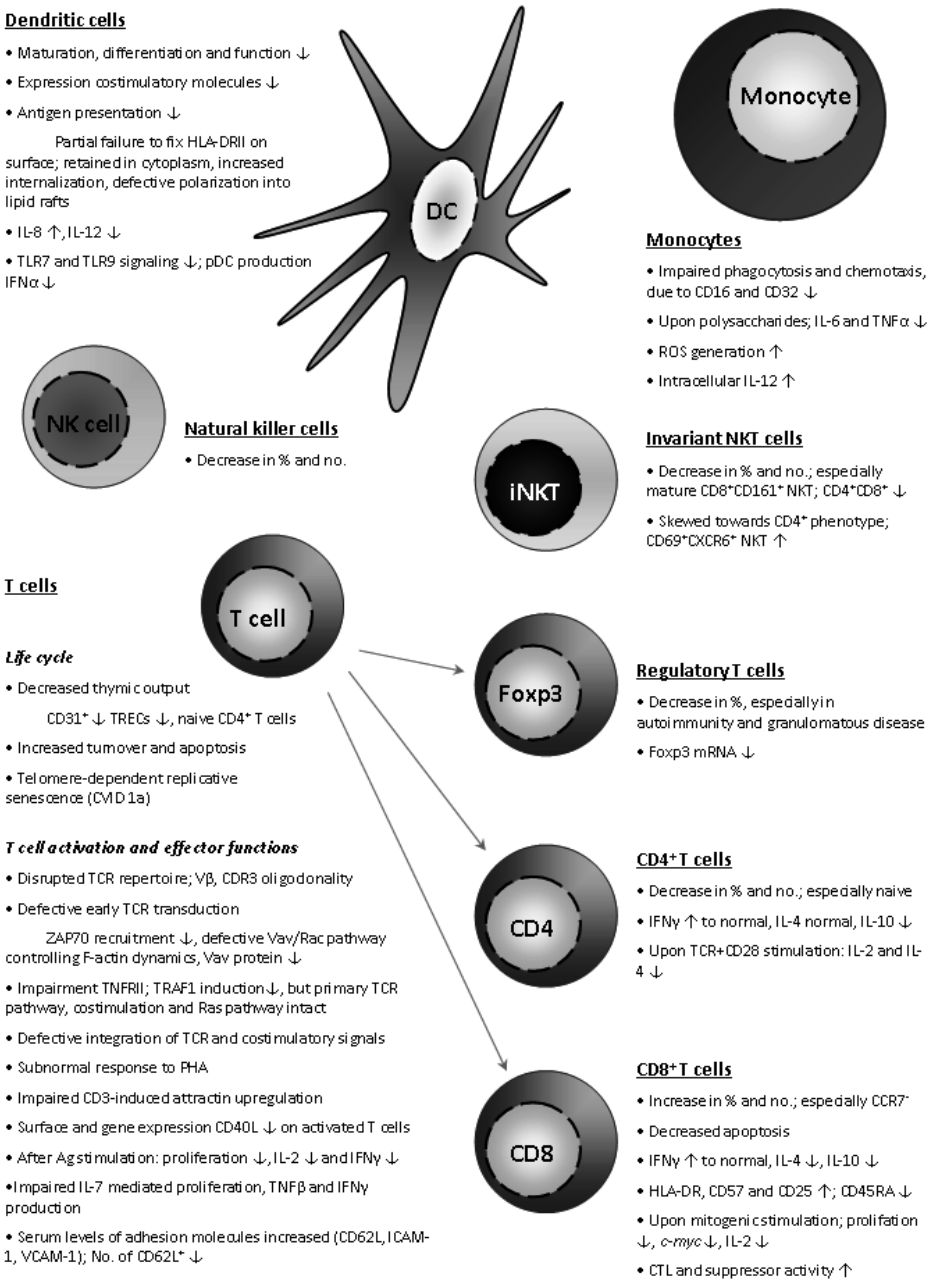

Figure 1: Additional leukocyte defects in CVID.
IL=interleukin; TLR=toll-like receptor; pDC=plasmacytoid dendritic cells; IFNα=interferon; TNF=tumor necrosis factor; ROS=reactive oxygen species; iNKT=invariant natural killer T cell; TREC=T cell receptor excision circle; TCR=T cell receptor; ZAP70=zeta-associated protein of 70kDa; TNFRII=tumor necrosis factor receptor II; TRAF1=TNF receptor associated factor 1 ; PHA=phytohemagglutinin; Ab=antibody; ICAM-1=intracellular adhesion molecule 1; VCAM=vascular cell adhesion molecule 1; CTL=cytotoxic T lymphocyte

Most of the data in this thesis were gathered in a pediatric CVID population. As their disease is usually in an early stage, there is a window of opportunity to study the development of complications. Especially in this young population, with a lengthy life expectancy, optimal complication management is essential. Additionally, from the etiological point of view, the immunopathogenesis of CVID may be more straightforwardly investigated in a population with less co-morbidity and associated use of immunosuppressive medication.

OUTLINE OF THE THESIS

Part I: Phenotypic and functional lymphocyte alterations in pediatric CVID disorders

In chapter 2-3, we explore whether pediatric CVID is comparable to adult CVID, and whether adult-based CVID classifications can be applied to the pediatric population. Chapter 4 summarizes current data on CVID etiology and proposes further research areas. In chapter 5 and 6, we investigate pathogenetic mechanisms involved in CVID. In chapter 5, early B cell activation is addressed, while chapter 6 illustrates the intricate study of CVID susceptibility genes in a family with TACI mutations.

Part IIa: Pulmonary complications in pediatric CVID disorders

The incidences and methods for detection of pulmonary complications in adult and pediatric CVID are reviewed in chapter 7, and obtained insights are applied in chapter 8. There, we define the incidence of pulmonary abnormalities in our cohort, based on a newly developed high-resolution computed tomography (HRCT) scoring system. This HRCT scoring system is compared with other detection methods in chapter 9, where we further explore clinical and immunological correlations with lung disease.

Part IIb: Pathogenesis of enteropathy in CVID and other antibody deficiencies

The third part of this thesis encompasses one of the most severe CVID-related complications, enteropathy. The case presented in chapter 10 exemplifies the difficulties in enteropathy treatment and suggests a potential pathogenetic mechanism of CVID-related enteropathy. In the next chapter, we study whether this hypothesis has some traction by reviewing the literature on persistent gastrointestinal virus infections in the immunocompromised host. Chapter 10 and 11 led to the initiation of a longitudinal observational study on gastrointestinal manifestations in pediatric CVID and related antibody deficiencies, which is currently running. Preliminary results of this study are presented in chapter 12.

At the conclusion of the thesis, a discussion and summary are presented.

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PART I

PHENOTYPIC AND FUNCTIONAL LYMPHOCYTE ALTERATIONS IN PEDIATRIC CVID DISORDERS

2

LYMPHOCYTE CHARACTERISTICS IN CHILDREN WITH COMMON VARIABLE IMMUNODEFICIENCY

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ABSTRACT

The diagnosis of common variable immunodeficiency (CVID) is reserved for patients who suffer from undefined B cell dysfunction. Division of the CVID population into subgroups enables research for underlying disease causes. We studied clinical features and lymphocyte characteristics in 38 children with CVID and compared them to 30 children with less severe antibody deficiencies (e.g. specific antibody deficiency combined with IgG subclass deficiency) and with 65 pediatric controls. Most pediatric immune phenotypes were comparable to adult CVID phenotypes, including a selective increase in newly formed B cells and a decrease in memory B cells and $CD4^+$ T cells. Eighteen percent of pediatric patients had a mutation in the *TNFRSF13B* gene, which requires further investigation. Finally, pediatric patients with decreased class-switched memory B cells had significantly more complications. A pediatric classification for CVID may enable prediction and early diagnosis of disease-related complications, and provide a framework for further etiologic research.

INTRODUCTION

Common variable immunodeficiency (CVID) is a primary immunodeficiency characterized by B cell dysfunction.[1;2] The diagnosis is based on decreased serum immunoglobulins (Ig) and a failure to produce antigen-specific antibodies in response to vaccinations or infections.[3] Besides recurrent infections, CVID patients have an increased tendency to develop autoimmunity, lymphoproliferative disease and malignancies.[4] Early prediction of complications is important as these disease complications cause severe morbidity, are associated with decreased survival and may require medical intervention. In addition to CVID, there is a spectrum of CVID-related antibody deficiencies that share clinical and immunological features. Patients diagnosed with selective antibody deficiency in combination with IgG subclass deficiency or symptomatic IgA deficiency may have as much recurrent infections as CVID patients, and may thus require immunoglobulin substitution therapy as well. Moreover, those diseases occasionally progress to CVID.[5;6]

The etiology of CVID is unknown for most patients. Mutations in the genes encoding the transmembrane activator and calcium-modulating cyclophilin ligand (CAML) interactor TACI,[7;8] inducible costimulator (ICOS),[9] CD19,[10] B cell activating factor receptor (BAFFR)[11] and CD81[12] have been described. Several biallelic and monoallelic variants of the *TNFRSF13B* gene encoding TACI have been described in CVID. Recently however, heterozygous variants were found in healthy individuals,[13] indicating that these variants are merely associated with increased susceptibility for CVID.[14;15]

Classifications divide the heterogeneous CVID population into more similar subgroups, to enable further etiological research and eventually give rise to personalized treatment options. Classifications of adult CVID patients have been ongoing: patients were classified based on flow cytometric markers of B cells,[16-18] T cells[19] or noninfectious complications.[20]

Immunological findings in pediatric CVID may exhibit important differences compared to CVID presenting in adults. Maturation of the immune system may influence the impact of gene mutations on clinical manifestations of CVID. Moreover, values for normality in this immature pediatric immune system are inherently different from those in adults.[21] For these reasons, there is need for evaluation of existing classifications in CVID. Here, we provide an overview of immunological characteristics of 68 children with CVID and related diseases. These data provide a framework for developing a pediatric CVID classification system.

METHODS

Patients and controls

We retrospectively analyzed data obtained between July 1995 and July 2008. Thirty-eight pediatric CVID patients of the Wilhelmina Children's Hospital in Utrecht, the Netherlands, were included. Diagnoses were made consistent with the European Society

for Immunodeficiencies (ESID) criteria.[22] Additionally, a group of 30 children with CVID-related diseases was investigated (selective antibody deficiency in combination with symptomatic IgA deficiency and/or IgG subclass deficiency, or 'probable CVID'). All CVID and CVID-like patients suffered from recurrent airway infections, and showed significant clinical improvement after initiation of immunoglobulin replacement therapy.

Our diagnostic protocol for primary immunodeficiencies consists of assessment of serum immunoglobulin titers, vaccination responses, T and B cell phenotyping and *in vitro* mitogenic and antigenic T cell proliferation responses. B cell proliferation assays were performed on a subset of patients. The most recent data were used in case of repetitive phenotyping. Clinical data were extracted from the medical files. We classified the children according to the classifications reported by EUROClass,[18] Giovannetti[19] and Chapel.[20] For comparison of phenotypical analyses, a control group of 65 healthy children was included. This cohort consisted of children without immune diseases who underwent elective surgery.[23]

Flow cytometry analysis

The T and B cell compartments were analyzed with four-color flow cytometry using whole blood and antibodies to CD3, CD45, CD27, CD4, CD8, HLA-DR, CD38, CD45RA and CD19, CD27, CD38, CD10, IgM, IgG, IgA, IgD, respectively as described previously.[23] All monoclonal antibodies were derived from Becton Dickinson and the polyclonal goat $F(ab')_2$ anti-human immunoglobulin antibodies from Southern Biotechnology Associates, Birmingham, Ala. After erythrocyte lysis, cells were analyzed on a FACS Calibur using CellQuest Pro data analysis software (both Becton Dickinson).

T and B lymphocyte functional assays

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque density gradient centrifugation and $1.8x10^5$/mL PBMC were cultured for 3 or 6 days in RH10 (RPMI1640 supplemented with L-Glutamate and 25mM HEPES (Gibco), containing 10% Human AB serum (Sanquin), Penicillin/Streptomycin (100 U/mL) (Invitrogen) and $6.0x10^{-5}$M β-mercaptoethanol (v/v) (Calbiochem)), and the following stimuli: phytohemagglutinin (5 µg/mL, Wellcome), concanavalin A (10 µg/mL, Calbiochem), tetanus toxoid (70Lf/mL, RIVM, the Netherlands), purified protein derivative (PPD) (13 µg/mL, Staten Serum Institute, Denmark), *Candida albicans* (7 µg/mL, Hal) and Diphtheria toxin (70 Lf/mL, RIVM). ^{3}H-Thymidine (1µCi /96well) was added 16 hours prior to harvesting. A stimulation index >3 was considered positive. Assay conditions were verified by a control sample run in parallel. The percentage of response was defined by the number of positive responses to a stimulus divided by the total number of tests.

For B cell differentiation assays, $4x10^5$ PBMC were cultured with either pokeweed mitogen (PWM, 3.5 µg/mL, Sigma) or *Staphylococcus Aureus* antigen (STA, 40 units/mL) and IL-2 (10 units/mL, Sanquin). After 7 days, cells were harvested, cytospins (10^5/sample) were prepared, air-dried, fixed with 95% EtOH/ 5% HAc and stained with FITC-conjugated anti-Ig, IgM, IgA or IgG (SBA). Ten fields of 50 cells were analyzed by fluorescence microscopy and the fraction of plasmablasts was calculated.

Molecular analysis of the *TNFRSF13B* gene

Genomic DNA was extracted from peripheral blood granulocytes with an autopure kit (Qiagen). Up to 300 µmol DNA was used in a TaqMan 5' nuclease assay. Primers and probes specific for the c.310T>C (p.C104R) variant, the c.542C>A (p.A181E) variant and their wildtype variants were designed with Primer Express software (Applied Biosystems), as shown in Appendix 1. Probes were labeled at the 5' end with either FAM or VIC as reporter dyes and at the 3' end with a minor groove binding (MGB) molecule (Applied Biosystems). The detection run consisted of a hot start at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. All assays were performed as 20 µL reactions using PCR Mastermix 2x (Applied Biosystems) in 96-wells plates using an ABI Prism 7900HT instrument (Applied Biosystems). We confirmed positive results by sequencing of the *TNFRSF13B* gene.

Statistical analysis

We corrected flow cytometry data for age and analyzed them with a Kruskal-Wallis one-way analysis of variance test. A p-value <0.05 was considered significant and post-hoc analyses were performed by Mann-Whitney *U* tests with Bonferroni corrections. Other statistical analyses were performed using Mann-Whitney *U* tests, Pearson's chi-square tests or Spearman's rank correlation coefficients with SPSS 15.0 for Windows.

RESULTS

Patient characteristics of CVID and CVID-like patients

We obtained data on 38 children with CVID and 30 children with CVID-like disease between 3 and 18 years *(table 1)*. There were seven pairs of siblings, and one family of three brothers with CVID. Four patients had immunocompromised family members not shown here, while the other 47 children represent sporadic cases. Most CVID patients were male (n=32, 84%) and the mean age at diagnosis was 5.5 years (range 0.9 tot 12.7 years). Laboratory data were collected at the time of diagnosis. In patients with an age of diagnosis <4 years, the diagnostic criterium of defective antibody production was re-confirmed after the age of 4 years. By definition, all CVID patients had decreased levels of IgG. Serum titers of IgA or IgM were decreased in 35 (92%) and 19 (51%) patients, respectively. Two patients presented with elevated levels of IgM, but showed normal CD40L expression upon stimulation. In general, hematological parameters were normal (data not shown). In the group of children with CVID-like disease, gender was equally distributed and the mean age at diagnosis was 6.1 years (range 2.2 to 14.2 years). Evidently, the serum immunoglobulin titers at diagnosis were significantly less affected than in CVID: 40%, 30% and 17% had a titer below two standard deviations for IgG, IgA and IgM, respectively (Pearson's chi-square test two-sided p<0.001, p<0.001 and p=0.009). Besides having recurrent infections, several patients had developed noninfectious symptoms related to CVID, such as autoimmune hemolytic anemia (AIHA) or enteropathy.

Table 1: Baseline characteristics of CVID and CVID-like patients

	CVID	CVID-like disease
Number of patients	38	30
Male/female	32/6 (84%/ 16%)	16/14 (53%/ 47%)
Age at diagnosis (years)	5.5 ± 2.5	6.1 ± 2.8
Bronchiectasis	1 (3%)	7 (23%)
Splenomegaly	1 (3%)	0 (0%)
Lymphadenopathy	2 (5%)	0 (0%)
Autoimmune disorders	3 (8%)	3 (10%)
Malignancies	1 (3%)	1 (3%)
Serum IgG (g/L)	4.14 ± 1.56	6.95 ± 2.57
High	0 (0%)	4 (13%)
Normal	0 (0%)	14 (47%)
Low	38 (100%)	12 (40%)
Serum IgA (g/L)	0.27 ± 0.25	0.53 ± 0.37
High	0 (0%)	0 (0%)
Normal	3 (8%)	21 (70%)
Low	35 (92%)	9 (30%)
Serum IgM (g/L)	0.55 ±0.45	0.86 ± 0.36
High	2 (5%)	1 (3%)
Normal	16 (43%)	24 (80%)
Low	19 (51%)	5 (17%)

Results depicted as mean ±1 standard deviation (SD) and categorically: 'high' (≥+2 SD), 'normal' (-2 SD to +2 SD), and 'low' (≤-2 SD) in absolute numbers and percentages

Alterations in B cell compartments in both CVID and CVID-like disease

To evaluate B cell development, peripheral $CD19^+$ B cells were analyzed for the presence of $IgD^+CD10^+CD38^{++}$ recent bone-marrow emigrants (RBE), $IgM^+IgD^+CD27^-CD10^-$ naive B cells, non-Ig class-switched $IgM^+IgD^+CD27^+$ memory B cells, IgG^+CD27^+ and IgA^+CD27^+ memory B cells and results were compared to those measured in healthy controls. Absolute numbers of the indicated B cell subpopulations are shown in figure 1. Both CVID and CVID-like patients had normal numbers of total B cells and $IgM^+IgD^+CD10^-CD27^-$ naive B cells, while $IgD^+CD10^+CD38^{++}$ RBE were significantly increased when compared to age-matched controls ($p<0.001$ for CVID; $p=0.015$ for CVID-like disease). Both the $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cell populations cells were decreased ($p<0.001$). Interestingly, the number of $IgM^+IgD^+CD27^+$ memory B cells was also decreased in both pediatric patient groups ($p<0.001$). A decrease in IgG^+ memory B cells was weakly correlated with a decrease in IgA^+ and IgM^+ memory B cells (Spearman's rank correlation: $r=0.5$, $p<0.001$ and $r=0.5$, $p=0.002$, respectively).

Comparison of relative numbers of B cell subsets with healthy controls yielded similar results: the percentages of total B cells, $IgD^+CD10^+CD38^{++}$ RBE and $IgM^+IgD^+CD27^-CD10^-$ naive B cells were increased, while all $CD27^+$ memory B cell subsets were decreased ($p<0.001$, data not shown). Comparison of CVID with CVID-like patients revealed no significant differences between the groups,

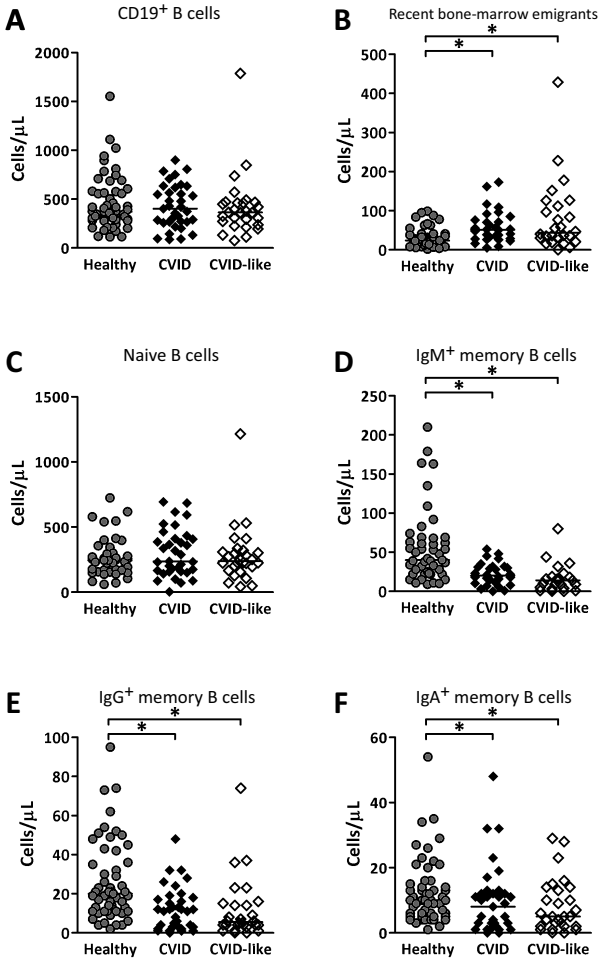

Figure 1: B cell subsets. Number (Cells/µL) of B lymphocyte subsets in healthy controls, children with CVID and CVID-like disease. Lines represent the median. The three groups were corrected for age before statistical comparison (Kruskal-Wallis, followed by Mann-Whitney *U* test with Bonferroni corrections). An asterisk indicates a p-value smaller than 0.05.

Alterations in CD4+ T helper cells in patients suffering from CVID, but not CVID-like disease

T cells were studied for potential T cell-mediated B cell dysfunction, and to investigate their role in the occurrence of complications. To this end, we divided $CD3^+$ T cells into $CD4^+$ and $CD8^+$ subsets and subsequently into $CD45RA^+CD27^+$ naive and non naive $CD45RA^+CD27^-$, $CD45RA^-CD27^+$ or $CD45RA^-CD27^-$ T cells (*figure 2*). CVID-like patients had no significant changes in T cell subsets, but children with CVID had decreased total $CD4^+$ T cells counts, associated with decreased counts of $CD4^+CD45RA^+CD27^+$ naive and non naive $CD4^+$ T cells (p=0.003, p=0.027 and p=0.003, respectively). The number of $CD45RA^+CD27^+$ naive $CD8^+$ T cells was also reduced (p=0.042). We found no significant differences directly between the patients groups. These deviations in T cell subsets were not associated with any of the reported B cell abnormalities.

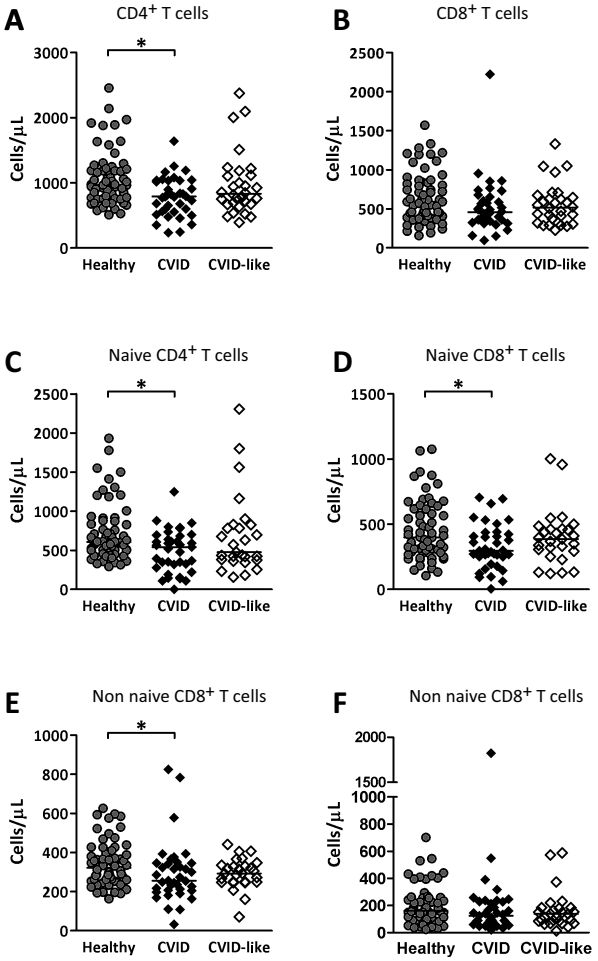

Figure 2: T cell subsets. Number (Cells/µL) of T lymphocyte subsets in healthy controls, children with CVID and CVID-like disease. Lines represent the median. The three groups were corrected for age before statistical comparison (Kruskal-Wallis, followed by Mann-Whitney *U* test with Bonferroni corrections). An asterisk indicates a p-value smaller than 0.05.

Impaired antigenic T cell responses and T cell-dependent B cell differentiation

We now asked whether lymphocyte populations were impaired in generating a functional immune response, or whether the decreased numbers did not affect their functional capacity. We addressed this question by assessing T cell proliferation with ^{3}H-thymidine incorporation assays. Except for two patients in the CVID-like group, all patients' T cell responses to mitogens were similar to those attained by healthy control T cells. The proliferation responses to antigens were partially decreased; approximately 70% of patients in both groups had suboptimal antigenic T cell responses compared with controls. Although not statistically significant, patients with disease-related complications seemed more severely affected in their antigenic T cells response than patients without any complications (mean response 53% *vs.* 71%, p=0.07).

Concurrently, we tested B cell differentiation into plasmablasts *in vitro* using two stimuli: *Staphylococcus Aureus* antigen (STA) combined with IL-2 for T cell-independent differentiation, or PWM for T cell-dependent differentiation, as shown in table 2. Patients of both groups showed a suboptimal differentiation upon stimulation with STA and IL-2 (46% in CVID, 20% in CVID-like disease). These impairments became more pronounced when T cells were required for plasmablast development; 67% of the CVID and 88% of the CVID-like patients did not develop normal amounts of plasmablasts. However, when plasmablasts developed, the majority produced all immunoglobulin isotypes.

Presence of TACI mutations in pediatric CVID patients

Deficiencies in lymphocyte compartments can be rather distinct between CVID patients, which likely originates from the variability in genes involved in the pathogenesis. We asked whether pediatric CVID patients have a similar prevalence of mutations in the *TNFRSF13B* gene encoding TACI as adult CVID patients, by screening for the two *TNFRSF13B* mutations that abrogate TACI function and significantly correlate with the prevalence of CVID: p.C104R[24] and p.A181E.[25] Of the fifty-one patients that were tested, nine (18%) had a heterozygous mutation (*table 3*). *TNFRSF13B* mutations could not be correlated with any clinical or immunological parameter.

Comparison with adult CVID classification systems

We classified all patients according to three classification systems, as shown in table 4.

Using the EUROClass classification,[18] CVID and CVID-like pediatric patients were similarly distributed, but the overall incidence of patients with a low percentage of

Table 2: *In vitro* B cell differentiation into plasmablasts in patients with CVID and CVID-like disease

	CVID (n=38)	CVID-like disease (n=30)
T cell-independent B cell differentiation with *S. Aureus* antigen (STA) and IL-2	Number tested: 28	Number tested: 20
Normal number *(percentage)*	15 (54%)	16 (80%)
Poor	8 (29%)	3 (15%)
Very poor	2 (7%)	1 (5%)
No differentiation	3 (11%)	0 (0%)
T cell-dependent B cell differentiation with pokeweed mitogen	Number tested: 24	Number tested: 17
Normal number *(percentage)*	8 (33%)	2 (12%)
Poor	6 (16%)	6 (35%)
Very poor	2 (8%)	3 (18%)
No differentiation	8 (33%)	6 (35%)
Expression of immunoglobulin classes	Number tested: 25	Number tested: 18
Three classes (IgG, IgA and IgM)	16 (64%)	14 (78%)
Two classes	7 (28%)	3 (17%)
One class	0 (0%)	1 (6%)
No immunoglobulins	2 (8%)	0 (0%)

Table 3: Clinical and immunological characteristics of patients with heterozygous TACI mutations

Patient	Mutation (heterozygous)	Diagnosis	Age at diagnosis (yrs)	Family	Gender[a]	Infections
1	A181E	CVID-like	3.4		M	Multiple pneumoniae, RSV bronchiolitis, gastrointestinal infections, 2x sepsis
2	C104R	CVID-like	4.7		M	Recurrent upper respiratory tract infections (URTI)
3	C104R	CVID	4.7		F	Sepsis after omphalitis, URTI, bronchitis,
4	A181E	CVID	6.7	Brother of pt 8	M	Recurrent URTI
5	C104R	CVID	5.2		M	Meningitis, pneumonia, frequent URTI and gastrointestinal infections
6	A181E	CVID	3.6	Brother of pt 7	M	Recurrent URTI
7	C104R	CVID	3.2	Brother of pt 6	M	Recurrent URTI, bronchitis
8	A181E	CVID	6.5	Brother of pt 4	M	Recurrent URTI
9	A181E and C104R	CVID	10.7		F	Persistent cytomegalovirus and norovirus infection

[a] M=male, F=female
[b] EUROClass classification: smB+ means >2% class switched memory B cells; smB-Tr^{norm} means ≤2% class switched memory B cells with <9% transitional B cells and smB-Tr^{high} means ≤2% switched memory B cells with ≥9% transitional B cells
[c] Autoimmune hemolytic anemia

	Non infectious sequelae	T cells	B cells	EURO Class classification[b]	Response to polysaccharide vaccines
	bronchiectases	Naive T cells increased, normal function	Decrease of all CD27$^+$ memory cells	smB+	Poor
		Increased naive T cells, activated phenotype, normal function	Normal phenotype	smB+	Moderate
		Naive T cells increased. No response to tetanus and diphtheria	Decreased IgG$^+$ and IgA$^+$ memory cells. No T cell-dependent differentiation	smB+	Poor
		Normal phenotype and function	Increased no and % of B cells; memory B cells decreased. Poor T cell-dependent differentiation	smB+	Moderate
		Normal phenotype and function	All CD27$^+$ memory B cells decreased. No T cell-dependent differentiation	smB-Tr^{high}	Poor
		Normal phenotype, no response to tetanus and diphtheria	Normal phenotype and differentiation	smB+	Very poor
		Normal phenotype, no response to tetanus and diphtheria	Normal phenotype, poor B cell differentiation of both types	smB+	Good initial response, but rapid titer decline
		Naive T cells increased, normal function	Low counts of B cells, normal differentiation	smB+	Poor
	AIHA[c], autoimmune nephritis, polycytic lung infiltration	T cell lymphopenia, no response to diphtheria and purified protein derivative (PPD)	Low number of B cells, decrease of all CD27$^+$ memory cells	smB-Tr^{high}	Very poor

class-switched memory B cells was lower than in adult CVID patients. We noticed a statistically significant relation between decreased class-switched memory B cells in combination with increased transitional B cells (smB-Tr^{high} group) and the occurrence of disease-related complications (p=0.001, Pearson's chi-square).

Subsequently, a correlation between naive CD4$^+$ T cells and clinical phenotype (disease severity, incidence of autoimmunity) as reported by Giovannetti *et al.*[19] was verified. Although we did find that naive CD4$^+$ T cells were significantly decreased, we were unable to relate this to a clinical phenotype. Thirdly, patients were classified using

Table 4: Frequencies (no.) and percentages (%) of children with CVID and CVID spectrum diseases classified according to the classifications of EUROClass,[18] Giovannetti[19] and Chapel. [20]

		CVID (n=38)		CVID-like disease (n=30)		Adult data literature[a]	
		No.	%	No.	%	No.	%
Giovannetti	<15% Naive CD4+ T cells	1	2.7%	0	0%	20	33.3%
	16-29% Naive CD4+ T cells	4	10.8%	0	0%	20	33.3%
	>30% Naive CD4+ T cells	32	86.5%	27	100%	20	33.3%
	Missing	1		3			
EUROClass	≤2% Switched memory B cells; ≥9% transitional B cells	8	22.2%	7	24.1%	25	9.6%
	≤2% Switched memory B cells; <9% transitional B cells	1	2.8%	1	3.5%	108	41.5%
	>2% Switched memory B cells	27	75%	21	72.4%	127	48.9%
	Missing	2		1			
Chapel	No complications[b]	33	86.8%	26	86.7%	159	47.6%
	1 Complication	4	10.5%	4	13.3%	118	35.3%
	2 Complications	1	2.6%	0	0%	42	12.6%
	3< Complications	0	0%	0	0%	15	4.5%

[a] Data were extracted from the referred articles
[b] Including: autoimmunity, polyclonal lymphocytic infiltration, enteropathy and malignancies

the method described by Chapel *et al*.[20] Seven patients had one complication; four of them where related to autoimmunity (celiac disease, systemic lupus erythematosus, or thyroiditis), one had lymphadenopathy and two patients suffered from a malignancy (acute lymphatic leukemia and a teratoma). Two patients had two complications each; one patient had idiopathic thrombocytopenic purpura (ITP), AIHA and enteropathy, while another child (patient 9, table 3) suffered from polyclonal lymphocytic infiltration and several autoimmune phenomena. These children were significantly more affected in their numbers of class-switched memory B cells than children without any disease-related complication (p=0.04 for IgG^+ and p=0.05 for IgA^+ memory B cells). Additionally, there was a trend towards more severely decreased serum levels of IgG and IgA at the time of diagnosis (for IgG: 4.0 *vs.* 5.6 g/L and for IgA: 0.23 *vs.* 0.40 g/L, both p=0.06) in patients with disease-related complications, while age, gender and diagnosis were similarly distributed.

DISCUSSION

Classifications for CVID subdivide the heterogeneous CVID patient population into distinct subcategories, with the aim to enable further etiological studies and to improve patient care. This study presents immunological data on a large cohort of pediatric CVID and CVID-like patients in tertiary care. We found that the immunological

abnormalities in children with CVID are comparable to those found in adults, including decreased class-switched memory B cells, increased recent bone-marrow emigrants and decreased CD4+ T helper cells. With these data, we show that the association between a decrease in class-switched memory B cells and the development of disease-related complications also holds true for pediatric CVID patients.

Presenting symptoms in pediatric patients were comparable to those in adults, with all patients reporting recurrent airway infections and an increased incidence of gastrointestinal infections. In B cells, lymphocyte phenotyping revealed decreased numbers of class-switched CD27+ memory cells in children with CVID and CVID-like diseases, while RBE were increased. Additionally, we observed a prominent decrease in memory IgM+ cells, previously only reported in a subgroup of adult CVID patients.[26] This decrease may be part of a general defect in B cell differentiation that is only observed in pediatric CVID, since adults have relatively more class-switched memory cells and rely less on their primary adaptive immune responses. Impaired B cell differentiation was observed in both CVID and CVID-like patients. There were no significant differences between these groups with respect to the decrease in B cell responses in T cell-dependent and -independent assays, implying that intrinsic B cell dysfunction as well as defective B-T interaction may play a causal role in pediatric CVID.

T cells may play an important role in the pathogenesis of CVID.[27-29] We analyzed CD4+ and CD8+ T cells, revealing a decrease primarily in absolute numbers of CD4+ T helper cells in pediatric CVID, but not in CVID-like patients. These findings could be the result of a decreased thymic output; alternatively, the patients' high infectious burden may accelerate T cell turnover and induce a secondary decrease in naive CD4+ T cells.[19;30;31] While we found differences between CVID and CVID-like patients with regard to cell counts of CD4+ T cells, T cell function was similar between groups. However, impaired antigenic T cell responses might predispose to the development of complications, an observation that requires further investigation.

The TACI gene is mutated in approximately 5-10% of adult CVID patients.[8;13;14;32] During maturation of the immune system, certain immune-related diseases (e.g. childhood allergies) disappear spontaneously, probably corroborated by age-related increases in memory B and T cell repertoires. In our cohort of CVID-like patients, the percentage of the p.C104R and the p.A181E mutations in TACI was similar to the prevalence described in adult CVID (2/23, 9%). However, in children with a complete CVID diagnosis, the percentage was surprisingly high (7/28, 25%). One could speculate that mutations in TACI predispose to a childhood (and temporary) form of CVID, or alternatively, that they invoke a more severe CVID phenotype, already manifesting at an earlier age. We can, however, not exclude the possibility that in our relatively small cohort, TACI mutations may be over-expressed due to e.g. the inclusion of several familial CVID cases. Thus, further research is required to investigate whether the CVID susceptibility gene *TNFRSF13B* may be influenced by maturation of the immune system.

Lastly, we verified whether adult classifications also apply to pediatric patients. Using the complication-based classification scheme by Chapel *et al.*, we found a lower

complication rate than was described in adults and in a recent pediatric study.[33] We confirmed a significant correlation between decreased numbers of class-switched memory B cells and the occurrence of disease complications, as shown previously in adult CVID patients.[16-18] Additionally, patients with a CVID-related complication had a trend towards lower IgG and IgA serum levels at diagnosis, a finding with potential predictive value. Taken together, some of the disease mechanisms that corroborate the development of clinical complications in adult CVID patients may also occur in pediatric CVID patients. Disease-related complications can be the presenting symptom of disease, but more often arise during follow-up. It is therefore plausible that the incidence of complications will rise in this cohort. Although recently reported data provide further insight into normal values for different subsets of B and T lymphocytes,[23] further studies are needed to define optimal cut-off levels for prediction of disease-related complications in pediatric CVID.

In conclusion, we present data enabling further development of a pediatric classification for CVID. Such a classification system will allow for prediction and early diagnosis of disease-related complications, as well as provide a framework for further etiologic research.

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Appendix 1: Primers and probes for CVID-related TNFRSF13B variants and their wildtypes

p.C104R in exon 3	
5′-primer	5′-TCTCCTGAGGGACTGCATCAG-3′
3′-primer	5′-GAGCTCTGGTGGAAGGTTCACT-3′
wildtype probe	5′ FAM-ACTTCTGTGAGAACAAGC-3′-MGB
mutant probe	5′ VIC-ACTTCCGTGAGAACAAGC-3′-MGB
p.A181E in exon 4	
5′-primer	5′- AGCCTAATGACGGGAAGAGAAGA -3′
3′-primer	5′-AAGACTTGGCCGGACTTTGAC-3′
wildtype probe	5′ FAM-CAGGCCACCGCCAC-3′-MGB
mutant probe	5′ VIC-CAGGCCACCTCCAC-3′-MGB

MGB=minor groove binding molecule

Appendix 2: Pediatric immunoglobulin reference values

	Reference values (g/L)		
Age (years)	**IgG**	**IgA**	**IgM**
0.5-1	2.6-15.2	0.16-0.98	0.17-1.2
1-2	2.6-13.9	0.19-1.1	0.10-0.87
2-3	4.3-13.0	0.19-2.3	0.21-0.87
3-6	5.2-13.4	0.55-2.2	0.24-1.8
6-9	5.2-14.3	0.54-2.5	0.28-1.9
9-12	5.2-15.6	0.62-3.0	0.13-1.6
12-16	5.2-15.6	0.70-3.6	0.28-2.4

3

CLINICAL COMPLICATIONS IN PEDIATRIC CVID ARE NOT RESTRICTED TO PATIENTS WITH SEVERELY REDUCED CLASS-SWITCHED MEMORY B CELLS

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Editor,

Yong *et al.* recently described immunologic and phenotypic findings in 45 pediatric CVID patients.[1] They correlated numbers of switched memory B cells to CVID-related immunologic complications by categorizing them into two groups: group I (switched memory B cells < 5 cells/μL) or group II (switched memory B cells ≥ 5 cells/μL). They observed that group I was significantly more affected in B- and T-cell numbers. Moreover, significant complications including meningitis, sepsis, autoimmune disease, gastrointestinal disease, bronchiectasis, pulmonary granulomatous disease, lymphadenopathy, splenomegaly and hematological malignancies were exclusively noticed in group I.

We recently published data on 68 children with CVID and related 'CVID-like' disorders and also concluded that children with reduced switched memory B cells had significantly more complications.[2] As validation of the cutoff level for switched memory B cells reported by Yong *et al.* was suggested, we evaluated their proposed cutoff levels in our pediatric population.

Seventy-one children (40 CVID, 31 CVID-like diseases) were re-evaluated. Fourteen (eight CVID) patients belonged to group I and 57 patients (32 CVID) to group II. Race, gender, immunoglobulin titers and age at diagnosis were similar to Yong *et al.*'s cohort. We confirmed that numbers of B cells, T cells and $CD4^+$ T cells were significantly lower in patient group I; in our cohort, the reduction of $CD8^+$ T cells was also significant (p=0.002).

In contrast to the published data, severe infections were not limited to group I patients *(table 1)*. Additionally, we found autoimmune complications, celiac disease and one patient with acute lymphatic leukemia in group II. Lastly, we investigated pulmonary complications in a subgroup of 54 patients using HRCT and found – among other abnormalities- a substantial prevalence of bronchiectasis in both groups (38-64%).[3]

Thus, we confirmed that pediatric CVID patients with < 5 cells/μL switched memory B cells have more abnormalities in other immunologic parameters and their prevalence of clinical complications is increased, pleading for more aggressive surveillance. However, higher numbers of switched memory B cells do not exclude severe complications, as we also found several severe infections and CVID-related complications in group II.

The differences in pulmonary findings could be result from our cohort screening using HRCT, which is a very sensitive detection method. Furthermore, the patients of group I were slightly older (mean age 15.4 yrs *vs.* 13.1 yrs, p=0.05), thus having a longer time span to develop complications. Low numbers of switched memory B cells were also found in CVID-like disease, and exclusion of CVID-like patients did not significantly change our findings.

In conclusion, the cutoff values used by Yong *et al.* may be valuable for classifying pediatric CVID; however, the development of complications remains a threat in both groups. Additional studies may be beneficial in the optimization of a pediatric classification system.

Table 1: Clinical complications in pediatric CVID.

	Number (%)	
Characteristic	**Group I (n=14, 20%)**	**Group II (n=57, 80%)**
Infectious		
Meningitis	2 (14%)	3 (5%)
Sepsis	2 (14%)	3 (5%)
Autoimmune		
Any autoimmune cytopenia	4 (29%)	1(2%)
Neutropenia	1 (7%)	1 (2%)
Hemolytic anemia	3 (21%)	0 (0%)
Immune thrombocytopenic purpura	3 (21%)	0 (0%)
Other (systemic lupus erythematodes, vitiligo, autoimmune thyroiditis, hepatitis and nephritis)	2 (14%)	2 (4%)
Gastrointestinal		
Celiac disease	0 (0%)	2 (4%)
Protein-losing enteropathy	1 (9%)	0 (0%)
Pulmonary		
Bronchiectasis	7/11 (64%)	14/37 (38%)
Granulomatous disease	2/11 (18%)	0 (0%)
Asthma	1/11 (9%)	4/37 (11%)
Oncologic		
Leukemia	0 (0%)	1 (2%)
Lymphatic		
Lymphadenopathy	2 (14%)	1 (2%)
Splenomegaly	1 (7%)	1 (2%)

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4

B-CELL DEFECTS IN COMMON VARIABLE IMMUNODEFICIENCY: BCR SIGNALING, PROTEIN CLUSTERING AND HARDWIRED GENE MUTATIONS

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ABSTRACT

Common variable immunodeficiency (CVID) is the most frequently diagnosed symptomatic primary immunodeficiency. CVID develops as a consequence of absence or malfunction of proteins involved with immunoglobulin production by plasma and memory B-cells. The last decade has brought us clarification of several genetic predispositions to the development of CVID. Despite considerable effort, however, for eighty-five percent of CVID patients, disease etiology remains undefined. We propose that in subsets of patients, CVID may involve defective assembly of protein complexes, which is crucial for example for B cell activation upon antigen triggering of the B cell receptor/co-receptor complex. Such defective protein-protein interactions may not be uncovered by standard gene sequencing methods, and may involve epigenetic or post-transcriptional regulation. In this review, we summarize recent developments in CVID research and propose additional approaches to the clarification of etiology of CVID patient groups, necessary for development of tailored treatment options.

INTRODUCTION

Humoral immunodeficiencies encompass the majority of primary immunodeficiency diseases and clinically manifest themselves primarily by recurrent bacterial infections of the respiratory tract.[1] The spectrum of antibody deficiencies is broad and ranges from asymptomatic IgA deficiency to severe infections in agammaglobulinemia patients. At an estimated prevalence of 1 per 25,000 Caucasians, common variable immunodeficiency (CVID) is the most frequent antibody deficiency that requires treatment.[2;3] Apart from recurrent infections, CVID patients have increased propensity to noninfectious disease-related complications, including autoimmune disorders, lymphoproliferative disease, enteropathy and hematological malignancies.[4] Since its first description in 1953,[5] medical interventions have reduced the severity of CVID-related morbidity and mortality, especially by prevention of infections. A major step forward in this respect was made with the introduction of immunoglobulin replacement therapy.[6] However, this treatment does not ameliorate the noninfectious complications as described above, inevitably meaning that to date, CVID remains a debilitating disease with considerable morbidity.

Despite continuous and intense investigations, the etiology of CVID remains incompletely understood. CVID is diagnosed in a heterogeneous group of patients with B-cell dysfunction, which may be the result of several unrelated genetic causes. The multigenetic base together with paucity of homogeneity in the CVID patient population makes etiologic research a convoluted endeavor, causing the majority of immunological studies to be descriptive rather than mechanistic in nature. To date, these studies have mostly been focused on B- and T-cell phenotype, expression of surface molecules involved in activation an proliferation of B cells, and associations between cell subsets and clinical phenotypes.[7;8] The latter finding facilitated the development of CVID classifications aimed to identify subgroups of patients with overlapping disease etiology, with similar risk profiles for the development of disease-related complications, or patients in need of comparable treatment regimens.[9-12]

In the past decade, significant progress has also been made in the identification of monogenetic defects, albeit in a modest fraction of the patient population. These defects include the transmembrane activator and CAML interactor (TACI),[13;14] CD19[15] and inducible costimulator (ICOS).[16] Mechanistic investigations were initiated with focus on distinct cell populations related to CVID development.[17-19] For example, Rakhmanov *et al.* discovered that human $CD21^{low}$ B cells resemble phenotypically the murine B cell subset called B-1 cells. As B-1 cells are an autoreactive subset, this finding thus provided possible insight in the increased incidence of autoimmunity in CVID patients characterized by high numbers of these B-1 cell-like B cells.[20] Lastly, subsets of CVID patients exhibit defects in B cell receptor (BCR)-mediated calcium signaling and in Toll-like receptor (TLR) signaling pathways.[21;22] Here, we focus on insights from recent studies that now imply B cell activation defects in the development of CVID.

ABROGATION OF TNF RECEPTOR PREASSEMBLY MAY LEAD TO A CVID PHENOTYPE

Oligomerization is an important feature of TNF receptors

The TNF receptor superfamily consists of cell surface receptors that are critical for lymphocyte development and function[23] and include p60 and p80 (TNFα receptor I and II, respectively), CD40, CD27, Fas/CD95, TACI[24] and B Cell Activating Factor Receptor (BAFFR). The majority of TNF receptors are transmembrane glycoproteins containing three extracellular cysteine-rich domains (CRDs). As a conserved characteristic, these receptors display ligand-independent self-assembly into homo-trimeric complexes[25;26] via the pre-ligand-binding assembly domain (PLAD).[27] The relevance of this trimerization domain is demonstrated via dominant-negative heterozygous mutations that associate with several human diseases.[26] The clinical relevance of TNF receptor preassembly was confirmed by findings in autoimmune lymphoproliferative syndrome (ALPS), a severe condition caused by mutations in the *Fas* gene. Since receptor trimerization is necessary for normal function and signaling of TNFR members, heterozygous mutations may more readily show a disease phenotype than heterozygous mutations in non-oligomeric proteins. In a subgroup of CVID patients, the disease has been attributed to dominant mutations in TACI that preclude normal function. Defective TACI function can be caused by interference with receptor oligomerization, as will next be discussed.

TACI defects in CVID

The first genetic defect in the TNFR superfamily elucidated in CVID was the *TNFRSF13B* gene encoding TACI.[13;14] TACI is expressed on monocytes,[28] dendritic cells,[29] and peripheral B cells. Its known main functions are the instigation of isotype class-switching, mediation of immunoglobulin production in response to type II T-independent antigens and negative regulation of B cell homeostasis. TACI has two CRDs;[24] CRD1 is responsible for ligand-independent preassembly into TACI trimers,[30] while CRD2 is the binding site for its ligands, a proliferation-inducing ligand (APRIL) [31] and BAFF.[32-34] TACI associates into trimeric complexes during assembly in the endoplasmic reticulum.[30] Additionally, TACI is displayed as oligomers on the B cell membrane.

TACI mutations were reported in CVID and involve inability for TACI to form oligomers or to bind ligands.[13;14] Heterozygous C104R and A181E TACI mutations were described that were not found in any of the 50 healthy controls.[13] The C104R mutated version of TACI no longer binds BAFF. In some patient B cells, stimulation with APRIL did not elicit isotype class-switching. Homozygosity with respect to S144X and C104R mutations resulted in loss of TACI function.[14] The C104R mutation is located in CRD1; C104R thus has a dominant-negative effect by abrogating oligomerization of TACI monomers.[30] A181E is located in the transmembrane domain, where it introduces a negative charge in the normally uncharged membrane-spanning region. The A181E TACI mutation alters both constitutive and ligand-induced NF-κB signaling, possibly

due to preclusion of normal receptor assembly by polar residues within the TACI transmembrane regions.[35] The impact of the A181E mutation was confirmed in a mouse model; its murine equivalent A144E abolished constitutive and ligand-induced TACI clustering, leading to disrupted signaling and impaired TACI-dependent B cell functions.[36]

The role of TACI in CVID was reconsidered when several mutations were identified in the healthy population; TACI was redefined as a susceptibility gene, rather than a disease-causing monogenetic defect.[37;38] To date, heterozygous C104R, A181E and arguably C204insertionA sequence variants constitute risk factors for CVID. A multicenter study detected TACI mutations in 50 hypogammaglobulinemic patients (8.9%), of which two were homozygous, 41 heterozygous and 7 compound heterozygous mutations.[39] Heterozygous mutations in C104R, A181E or I87N were found in 2% of the healthy controls. Although at a much lower rate than heterozygous TACI mutations, asymptomatic patient family members could carry biallelic mutations as well, complicating elucidation of the exact role of TACI in CVID. Apart from being a susceptibility gene for the development of CVID, heterozygous TACI mutations also predispose to autoimmunity and lymphoid hyperplasia in CVID, but immune and clinical phenotypes remain diverse.[40]

BAFFR and other TNFR defects in CVID

The ligands for TACI can bind two additional known receptors: B cell maturation antigen (BCMA)[41;42] and BAFFR. BAFFR is widely expressed by all B cells except bone marrow plasma cells.[43;44] Upon binding of BAFF, BAFFR transmits survival signals and thereby positively regulates B cell survival. A homozygous deletion within the BAFFR-encoding *TNFRSF13C* gene was described in two related individuals, causing a partial deletion of the BAFFR transmembrane region, thereby abolishing BAFFR expression.[45] As a result, development and homeostasis of B cells in follicles and marginal zones were impaired, and memory B cells were low. IgA responses remained normal and T cell-independent responses against polysaccharides were abrogated. Nonetheless, the clinical phenotype of the affected individuals was not as severe as one would expect based upon these immunological features. One individual displayed characteristic recurrent lower respiratory tract infections, but no autoimmunity, and was not diagnosed with CVID until the age of 57. Of note, his sibling had an unremarkable medical history until the age of 70, showing that BAFFR deficiency does not inevitably lead to overt immunodeficiency.

Furthermore, DNA samples from 49 families consisting of at least two related CVID patients and 50 sporadic patients were sequenced for the occurrence of mutations in the genes encoding APRIL and BCMA. No mutations were found.[46] Deficiency of CD27, a TNFR family member expressed on memory B cells and on T cells was recently identified in a patient with a medical history of chronic active Epstein-Barr virus infection, hypogammaglobulinemia and abnormal T-cell dependent B cell responses. (Van Montfrans *et al.*, submitted) In conclusion, heterozygous alterations in TNFR family members may predispose to the development of CVID, but should not be considered as disease-causing monogenetic defects. Homozygous and compound

heterozygous alterations in the genes encoding these receptors presumably cause a stronger association with CVID, but a small number of healthy carriers have been described.

HYPOGAMMAGLOBULINEMIA DUE TO HOMOZYGOUS MONOGENETIC DEFECTS IN THE BCR/CO-RECEPTOR COMPLEX

Signaling threshold depends on assembly of the B cell receptor with its co-receptor complex

Reorganization of membrane-expressed receptors is now established as being pivotal to lymphocyte activation.[47] Until recently, the widely accepted model for antigen-specific activation of the B cell receptor (BCR) involved crosslinking of multiple monomeric BCRs by antigen. A new view of BCR-mediated activation is based on the demonstration of autoinhibited BCR oligomers on resting B cells that upon activation acquire an open, antigen-receptive conformation; a dissociation-activation model.[48] While appealing, it is too early for the new model to have gained experimental support and substantial intellectual traction. As both models support, cognate antigen binding by BCRs induces signaling of the BCR via the associated Igα/Igβ heterodimer, that both contain immunoreceptor tyrosine-based activation motifs (ITAMs).[49-51] Src-family kinases Fyn, Blk and Lyn are thereby recruited, Bruton's tyrosine kinase (Btk) is phosphorylated, and phospholipase Cγ2 is activated, which in turn facilitates the rapid release of calcium from the endoplasmic reticulum into the cytoplasm.[52-56] As a consequence, a sustained calcium entry into the cell occurs via plasma membrane associated store-operated calcium entry (SOCE) channels, which is necessary for full stimulation of B cells that results in plasma cell differentiation and immunoglobulin secretion.[57]

Antigen-mediated signaling is enhanced by simultaneous binding of antigen to the BCR co-receptor complex. The BCR co-receptor complex consists of CD21 (CR2, complement receptor 2), CD19, CD81, CD225 and possibly other molecules. Complexes of antigen bound by the large cleavage products of C3 (i.e., C3d and C3b) can therefore crosslink the BCR and CD21 co-receptor. CD21 is laterally associated with CD19 and upon antigen ligation, the cytoplasmic CD19 tail is phosphorylated, resulting in binding and signal propagation via Src family kinases. Thus, BCR association with the co-receptor complex augments antigen-mediated signaling.[58;59]

Monogenetic defects in CVID and agammaglobulinemia

A small number of CVID patients with homozygous mutations in genes related to BCR signaling have been identified. These genetic defect all result in defective BCR co-receptor complex formation, and thus result in overlapping clinical phenotypes *(table 1)*. First, four patients were found to have a mutated *CD19* gene, resulting in

premature stop codons and the deletion of the cytoplasmic tail of CD19.[15] B cells were present, but expression of CD19 (and CD21) was marginal to absent. $CD27^+$ memory B cells and $CD5^+$ B cells were decreased and BCR-mediated activation was impaired, as was deducted from *in vitro* stimulation experiments. The antibody response of these patients to rabies vaccination was suboptimal.

One patient was identified having a mutation in the *CD81* gene, resulting in a complete CD81 protein deficiency. This patient, whose clinical features included hypogammaglobulinemia and nephropathy, showed absent CD19 surface expression, impaired responses upon BCR stimulation and reduced counts of memory B cells.[60] The first human CD21 deficiency has also recently been described.[61] And finally, human CD20 deficiency was documented to result in impaired T cell-independent antibody responses and hypogammaglobulinemia.[62] These data support the recently identified lateral membrane association of CD20 to the BCR.[63-65]

B cell receptor signaling defects were found in antibody deficiencies related to CVID, such as X-linked agammaglobulinemia (XLA). XLA is a humoral immunodeficiency in which mutations in the *Btk* gene result in negligent numbers of peripheral B cells and paucity of immunoglobulins.[66-68] Mutations in μ heavy chain,[69] BLNK,[70] Igα[71;72] and Igβ[73;74] are responsible for a resembling agammaglobulinemic phenotype. These findings indicate that molecules of the BCR signaling cascade are pivotal for B cell development and homeostasis, and for subsequent immunoglobulin production. The severity of immunodeficiency is mainly dependent on the consequence of the mutation on protein function and its location within the BCR signaling cascade. BCR proximal signaling defects can result in an early B cell developmental block and agammaglobulinemia, while downstream defects induce an immunodeficiency characterized by the presence of B cells, but with a variable degree of abnormal differentiation and function.

Mechanistic defects in B cell receptor signaling

Besides monogenetic defects, BCR-related mechanistic alterations were identified in subgroups of CVID patients. BCR triggering-induced calcium mobilization was used as a read-out for early BCR-mediated activation and was decreased in a subgroup of CVID patients, corresponding to class Ia of the Freiburg classification.[21] Patients in this group are characterized by a severe reduction in class-switched memory B cells and the expansion of innate-like $CD21^{low}$ B cells, and are prone to develop CVID-related complications.[10] Calcium mobilization defects were independent of constitutional differences in the B cell compartment. The authors hypothesized the defect to be at the level of the B cell plasma membrane and suggested a link with inhibitory sialic acid-binding immunoglobulin-like lectin (SIGLEC) receptor CD22. In our pediatric CVID cohort, we confirmed these calcium signaling alterations, although they were not associated with a particular CVID classification.(our unpublished observations)

Upon BCR crosslinking, CVID B cells exhibit a considerable reduction in tyrosine phosphorylation of relevant molecules compared to normal.[75] Moreover, the fusion of unaffected mouse plasma membrane vesicles into the plasma membrane of CVID B cells restored phosphorylation and immunoglobulin production in these patients,

Table 1: A selection of CVID-associated proteins known to require oligomerization for normal function

			Phenotype		
Deficiency	**Mutation**	**Oligomerization**	**Total B cells**	**Transitional B cells**	**Memory B cells**
BCR signaling					
CD19	Cytoplasmic tail	Required for binding multiple cytoplasmic proteins (Lyn, Vav, PLCy2, Grb2, and p85 subunit of PI3-K.	Normal	Normal	Low
CD20	Splice mutant causing abolished expression	Lateral association with BCR-complex.	Normal	Unknown	Low
CD81	Cryptic splice site mutation that cause abolished expression	Complex formation with CD19, CD21, and CD225; essential for lowering activation threshold.	Normal	Low	Low
TNFR superfamily					
TACI	C104R: CRD1 A181E: transmembrane-domain	C104R: CRD1 required for oligomerization A181E: required for receptor clustering in PM, essential for downstream signalling.	C104R: slightly increased A181E: low	Normal	Normal
BAFF-R	Transmembrane-domain	Precluding BAFF-R expression.	Low	Increased	Low
TLR family					
TLR 7	No mutations identified	Oligomerization for ligand binding.	Unknown	Unknown	Unknown
TLR 9	No mutations identified	Oligomerization for ligand binding.	Unknown	Unknown	Unknown

Phenotype					
IgM levels	**IgG levels**	**IgA levels**	**Thymus-dependent vaccination response**	**BCR-mediated calcium response**	**Reference**
Decreased	Low	Decreased	Decreased	Impaired	Van Zelm *et al.* [15] Kanegane *et al.* [108]
Normal	Low	Normal	Normal	Normal	Kuijpers *et al.*[62]
Normal	Low	Decreased	Impaired	Impaired	Van Zelm *et al.*[60]
Normal	Now	Now	Mice: Normal Human: Unknown	Normal	Castigli *et al.*[13] Salzer *et al.*[14] Garibyan *et al.*[30] Salzer *et al.*[39]; Own unpublished observations
Low	Almost absent	Normal	Normal	Unknown	Warnatz *et al.*[45]
Normal	Decreased	Decreased	Decreased	Unknown	Yu *et al.*[22]
Normal	Decreased	Decreased	Decreased	Unknown	Yu *et al.*[22] Cunningham-Rundles *et al.*[86]

suggesting the presence of an early signal transduction defect located in the B cell plasma membrane.[75] Others have described impaired BCR-mediated B cells effector functions, including defective upregulation of costimulatory molecules (CD70, CD86) [76;77] and decreased immunoglobulin production.[78] Although these data still require genetic support, their cumulative cell biology-based results imply a significant role of an aberrant BCR pathway in CVID.

FUNCTIONAL BUT NOT GENETIC DEFECTS IN TOLL-LIKE RECEPTORS IN CVID

Toll-like receptor signaling, a synopsis

TLRs recognize pathogen-associated molecular patterns (PAMPs) and play crucial roles in early, innate host defense against invading pathogens. TLRs are differentially expressed on dendritic cells, monocytes and B cells, and dependent on the type of TLR and likely availability of ligand, localization is at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) or on endosomal membranes (TLR3, TLR7 and TLR9).[79] Intracellularly, TLRs have a conserved region of about 200 amino acids, known as the Toll/IL-1R (TIR) domain, as this domain is shared with the IL-1 receptors. The TIR domain is essential for subsequent cytoplasmic signaling. Upon ligation, TLRs undergo dimerization and conformational changes in order to allow recruitment of downstream signaling molecules. The TIR domain associates with myeloid differentiation primary-response protein 88 (MyD88), which in turn recruits IL-1R-associated kinase 4 (IRAK4).[80;81] Subsequently, IRAK1 is attracted, phosphorylated by IRAK4 and TNFR-associated factor 6 (TRAF6) next associates with the phosphorylated IRAK1. This complex eventually allows NF-κB to translocate to the nucleus and induce the expression of its target genes.[82] In addition, there are MyD88-independent pathways of TLR signaling not addressed here. (A full description of TLR signaling pathways falls outside the scope of this review).

TLRs generally form homo-dimers, but selective members can hetero-dimerize with other TLRs as well (e.g. TLR2-6, TLR1-2) and as such expand their recognition repertoire.[83] Additionally, TLRs may require clustering with other molecules; e.g., the lipopolysaccharide (LPS)/LPS-binding protein (LBP) complex binds to CD14, enabling LPS/CD14 to associate with TLR4. TLR expression appears related to the developmental stage of the B cells; TLR6, TLR7, TLR9 and TLR10 are highly expressed on memory B cells, but low to undetectable in naive B cells. However, BCR stimulation may upregulate TLR expression in naive B cells.[84] TLRs play a role in B cell proliferation, differentiation and class-switch recombination.[85]

TLR signaling defects in CVID B cells

Considering their role in B cell function, TLRs are relevant research candidates in CVID. TLR9 is highly expressed on memory B cells and binds to unmethylated CpG dinucleotides, a strong natural bacteria-associated ligand. Expression of TLR9 was

decreased in CVID B cells. B cell TLR9 stimulation by CpG caused defective upregulation of CD86 and activation-induced cytidine deaminase (AID) mRNA, and decreased production of IL-6, IL-10, IgG and IgA.[86] The defects were more pronounced when combined TLR9 and BCR-triggering was performed: CD86 upregulation and B cell proliferation were markedly reduced in CVID patients upon stimulation with CpG and anti-IgM antibody-mediated BCR crosslinking.[86;87] Second, defects were described in the related endosomal RNA-binding TLR7. Stimulation with synthetic ligands loxoribine (TLR7) and CL097 (TLR7/8) inefficiently induced B proliferation and differentiation in CVID. Immunoglobulin production and AID mRNA upregulation were defective. These deficiencies were partly reversed by addition of IFNα, which is normally produced by plasmacytoid dendritic cells upon TLR7 ligation.[22] Alterations were not B cell restricted, but were also observed in CVID plasmacytoid dendritic cells.[22]

Taken together, defects in TLR signaling may play a substantial role in the pathogenesis of CVID. However, it remains uncertain whether this is an intrinsic molecular defect, or an extrinsic defect that involves interference with other pathways, including BCR signaling. We propose that the oligomerization of receptors or association of protein involved with B cell activation is probably involved. To date, genetic defects have not been identified although CVID patients have been screened; no mutations or polymorphisms were found in the *TLR9* gene[86] or its promoter.[88]

PROSPECTS

Multiple genetic and mechanistic defects related to B cell surface receptors in CVID were described in the last few years. That surface receptors were first addressed as candidates for B cell dysfunction in CVID is rational from a mechanistic point of view, as receptors provide the first contact with the extracellular environment and initiate all sequential B cell processes. Concomitantly, they are of importance in early B cell development and selection.[89]

Although the molecular discoveries provide insight in the mechanistic alterations in CVID, they provide a diagnosis in only a fraction of the patient population. CVID is variable as the name suggests and the etiology of CVID remains a conundrum in the majority of patients. In this regard, several problems are encountered.

First, although monogenetic defects have been identified, they usually represent only sporadic cases, mostly originating from consanguineous families. Their number remains low, in particular when taking into account the extensive screening efforts that have been performed on numerous patients for candidate genes related to B cell development and function.[46;90]

Second, the plasticity of the immune system and the consequent redundancy for most of its molecules is clearly beneficial for the maintenance of general health, but may hamper the search for disease-causing mutations. Even mutations in relevant genes that lead to immunodeficiency in some individuals do not necessarily cause a clinically overt CVID phenotype in others. This could be related to differences in penetrance or epigenetic regulation of gene expression.[91] The recently expanding

interest in epigenetics indicates the potential importance of alterations of post-translational modification state of proteins, which may change degradation or activation of the affected protein.

An alternative hypothesis is the influence of supplementary genetic variations between patients and asymptomatic siblings. As we anticipate that screening approaches that cover the entire genome for potential variations will yield a wealth of new CVID-associated genes, we believe that mechanistic cell biological studies may narrow down the defective proteins within affected B cells. However, former studies indicate that it is extremely challenging to link mechanistic defects to a genetic base in this heterogeneous patient population.[21;22] The interplay between different receptors is robust and complex.

TLR signaling is at several levels intermingled with BCR signaling. BCR stimulation induces the upregulation of TLRs on naive B cells.[21;84] BCR/TLR crosslinking can occur simultaneously by immune complexes such as in systemic lupus erythematosus.[92;93] Thus, a BCR defect will negatively influence TLR signaling and *vice versa*. Furthermore, TLR signaling is linked with TACI and BAFFR pathways;[94-96] He *et al*. moreover recently showed that TACI contains a conserved motif which binds adaptor MyD88, and thus employs the TLR pathway via a TIR-independent route.[97]

Taking into account these linkages in signaling pathways and the fact that CVID is multifactorial, it is plausible that for instance heterozygous TACI mutations in combination with a (minor) TLR signaling-related gene defect will lead to a CVID phenotype (whereas asymptomatic TACI mutant siblings have no additional genetic defect). Extended fundamental knowledge regarding the receptor pathway signaling interplay, in combination with precarious selection of patients based on their clinical and immunological features for further screening, will facilitate the identification of additional defects in CVID.

Taken together, we believe that the approach of genetic screening for candidate genes may not yield many novel genes in CVID patients, nor will isolated functional studies as currently performed be able to identify molecular defects. Ideally, a combination of these approaches should be performed, by investing in screening methods for target genes involved in basic functional (predominantly B cell-intrinsic) mechanisms. For such a modified screening approach, the investigation of surface receptor clustering, by combination of live cell confocal visualization techniques, flow cytometry and immunoprecipitation/blotting techniques, we consider an interesting candidate mechanism for two reasons.

First, as mentioned above and elsewhere, oligomerization is crucial for receptor function. Defective oligomerization impacts on functional quality, even in the presence of perfectly functional monomers, by means of dominant-negative interference; the C104R mutation in TACI provides an example for this situation. Relevant B cell surface receptors need dimerization or oligomerization in order to transduce signals. Therefore, an alternative possibility is that the intracellular association is defective; recruitment and hence interaction of adaptor proteins with the receptor may be impaired, eventually resulting in signaling defects and consequent defects in antibody class-switch recombination or other more overt CVID consequences.

Second, there is clinical evidence that dysfunctional receptor clustering, due to defective cytoskeleton rearrangements causes severe immunodeficiency disorders. This is demonstrated by at least two diseases: Wiskott-Aldrich syndrome (WAS) and DOCK8 deficiency. WAS is a rare X-linked primary immunodeficiency complicated by thrombopenia and eczema, due to loss-of-function mutations in the Wiskott-Aldrich syndrome protein (*WASP*) gene.[98] WASP regulates actin cytoskeleton reorganization by stimulation of ARP2-ARP3 mediated actin polymerization and has an expression restricted to hematopoietic cell lineages. As a result, WASP deficiency hinders immunological synapse formation in response to antigen receptor stimulation,[99;100] affecting T cell and arguably B cell activation and thus contributing to the immunodeficiency.[100-103]

Recently, mutations were found in the gene encoding dedicator of cytokinesis 8 protein (*DOCK8*)[104] in patients with a previously undefined combined immunodeficiency or atypical autosomal recessive hyper IgE syndrome.[105;106] All patients suffered from recurrent respiratory tract infections and extensive cutaneous viral infections, and many developed virus-related cutaneous dysplasia. Immunological features included T and B cell lymphopenia, eosinophilia, defective $CD8^+$ T cell proliferation and impaired antibody responses. DOCK8 is a Rac/Cdc42 GTP-exchange factor (GEF), a regulator of cytoskeleton rearrangement. DOCK8 is thought to have a relatively specialized role in adaptive immunity. Indeed, *DOCK8* mutated mouse B cells were unable to form marginal zone B cells and had diminished affinity maturation, due to poor survival of these cells. The defects were caused by impairment in immunological synapse formation; mutant B cells were unable to cluster ICAM-1 into a peripheral supramolecular activation cluster (SMAC).[107] These findings indicate that the organization of the immunological synapse, including receptor oligomerization, is critical for B cell subset survival.

The described examples of primary immune deficiencies clearly demonstrate the importance of synapse formation and membrane rearrangements for lymphocyte function. Possibly, other molecules involved in these rearrangements are affected in CVID; this idea is supported by the observation of defective expression of Vav, another member of the GEF family, and a subsequent impaired actin reorganization in CVID T cells.[17]

In conclusion, the elucidation of molecular defects in CVID remains a challenge and requires especially now, the continuous joint efforts of clinical and basic researchers with immunological, cell biological and genetic backgrounds.

Etiologic research should be extended by exploring mechanistic and functional lymphocyte defects in precariously selected patients. However, the approach remains limited by the current knowledge regarding the molecular basics of those basic mechanisms in B cells. Of particular interest are mechanisms that limited to B cells or adaptive immunity, as CVID patients in general have no other tissue problems. Subdividing patients based on phenotypic and functional B cell characteristics may facilitate and enhance the success rate of revealing novel defects in this complex disorder.

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5

DEFECTIVE CALCIUM SIGNALING AND DISRUPTED CD20-B CELL RECEPTOR DISSOCIATION IN COMMON VARIABLE IMMUNODEFICIENCY DISORDERS

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Submitted

ABSTRACT

Background: B cells of patients with common variable immunodeficiency (CVID) disorders display impairment in production of Ig-class-switched antibodies, possibly contributed by defects in early B cell activation. On resting B cells, B cell antigen receptors (BCRs) are organized in oligomers that are signaling inactive. Their triggering by cognate antigen causes the lateral reorganization of BCR and associated proteins into signalosomes, resulting in BCR-activated calcium entry. In resting cells, the B cell surface antigen CD20 is associated with the BCR, but dissociates upon signalosome formation.

Objective: We sought to determine whether CD20 dissociation from the BCR during early B cell activation may contribute to the development of CVID disorders.

Methods: We evaluated BCR signalosome formation, internalization and signaling in primary B cells of pediatric CVID disorder patients and healthy controls.

Results: We show that in a substantial number of pediatric CVID disorder patients, B cells exhibit significant deficits in BCR triggering-mediated calcium entry in the cytosol, which correlates directly with impaired plasmablast differentiation *in vitro*. These alterations did not originate from upregulation of CD22 or defects in calcium channels, and did not involve gene mutations in *PLCγ2* or *Btk*. Instead, CVID disorder B cells exhibited reduced BCR dissociation from CD20. BCR or CD20 crosslinking-induced less BCR internalization, and antibody-mediated CD20 triggering elicited less BCR downstream signaling as measured by secondary fluxes.

Conclusions: We propose that CD20 dissociation from the BCR signalosome is pivotal to BCR-mediated calcium mobilization in the cytosol. Defects in CD20/BCR signalosome conformation may predispose to the spectrum of CVID disorders.

INTRODUCTION

Common variable immunodeficiency (CVID) is a heterogeneous immunodeficiency characterized by defective antibody production[1] and CVID-related complications, including autoimmunity and lymphoproliferation.[2;3] Disease etiology remains unknown for 90% of patients.[4] Nearly ten percent of patients harbor mutations in transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) that are CVID associated but are not sufficient to cause CVID disease,[5-7] while in sporadic families mutations have been described in inducible T cell co-stimulator (ICOS),[8] CD19,[9] and B cell activation factor receptor (BAFFR).[9;10] Deficiency of CD81,[11] CD21[12], CD20[13] led to CVID-like B cell disorders with variable grades of hypogammaglobulinemia and antibody synthesis deficits. Mutations in the BCR co-receptor complex, combined with abrogated B cell effector functions, suggested the possibility of defective B cell activation in some patients with CVID or CVID-like disorders.

In the resting state, B cells express up to 120,000 signaling inactive B cell receptors (BCR).[14] Ligation of cognate antigen induces the reorganization of signaling active BCR oligomers, or signalosomes, that provoke elevation of intracellular calcium ion concentrations[15]. The BCR associates with CD79α/β proteins, that each contain immuno receptor tyrosine-based activation motif (ITAMs) for transmitting signals through binding of Src-family kinases. Binding of the co-receptor complex composed of CD19, CD21, CD81 and associated proteins, results in CD19 phosphorylation and recruitment of Src-family tyrosine kinases and PI-3-kinase. This results in activation of phospholipase Cγ2 (PLCγ2), which via generation of inositol 1,4,5-trisphosphate 3 (IP_3) and binding to IP_3 receptors at the endoplasmic reticulum (ER) membrane facilitates calcium release from ER stores into the cytoplasm. Consequently, calcium enters the cell via plasma membrane-associated store-operated calcium entry (SOCE) channels. We proposed that early defects in BCR-mediated calcium signaling may cause B cell dysfunction in CVID disorders. We investigated CD20 that is almost exclusively expressed on B cells.[16] While its role in B cell function is not fully clear, CD20 is a direct regulator or component of a calcium channel.[17;18] CD20 forms homo-oligomers that physically associate with the BCR,[19] and CD20 antibody treatment induces BCR-mediated signal transduction, including calcium flux and resulting in similar transcription patterns as BCR triggering.[20;21] Following BCR ligation, CD20 oligomers dissociate and remain on the cell surface, while the BCR and its accessory molecules are endocytosed.[19;22] CD20 deficiency in humans causes CVID-like disease.[13] Furthermore, B cells of CVID patients of the Ia subgroup of the Freiburg CVID classification, exhibit defects in calcium influx induced by BCR signaling.[12]

We here report defective BCR-mediated calcium signaling in B cells of numerous pediatric CVID disorder patients. Afflicted CVID disorder patients exhibited a reduction in cytosolic calcium mobilization upon BCR cross-linking in comparison to healthy controls. Rates of calcium mobilization in fresh CVID disorder B cells directly correlated with B cell differentiation efficiency *in vitro*. While it had been known that B cell activation induces plasma membrane dissociation of the BCR from the CD20 for induction of calcium fluxes, this phenomenon had not yet been associated with a

human disease. We show in our cohort of CVID disorders, defects in BCR internalization and calcium mobilization when induced by crosslinking of either CD20 or BCR. Thus, in selected patients, CVID disorders may involve defects in dissociation of the BCR from surface-retained CD20 during BCR-mediated B cell activation.

MATERIALS AND METHODS

Patients and healthy donors

Forty-two pediatric patients (mean age 12 years, range [4 -18]) with CVID disorders, fourteen healthy children (9 yrs [4-13]) and 23 adult volunteers (29 yrs [23-40]) were included in this study; detailed information is available in the Supplementary Methods. This study was approved by the institutional review board and informed consent was obtained.

Flow cytometry

Besides previously described B cell phenotyping,[23;24] peripheral blood mononuclear cells (PBMC) were stained with antibodies to CD19, CD20, CD21, CD22, CD27, CD38, CD45 (all Becton Dickinson), IgM $F(ab')_2$ and Igλ $F(ab')_2$ (Southern Biotechnology Associates, Birmingham, AL). Samples were acquired on a FACS Canto II Flow Cytometer and analyzed using FACS Diva software (both Becton Dickinson).

Flow cytometric analysis of calcium flux

One million fresh PBMC were isolated and loaded directly with 4 μM Fluo-3, 10 μM Fura Red (Invitrogen, Carlsbad, CA) and later, CD19-APC was added. Cells were washed twice and resuspended in Hank's balanced salt solution (HBSS) supplemented with fetal calf serum (FCS). Baseline cytosolic calcium levels were measured on a FACS Canto II flow cytometer. Next, 20 μg/mL anti-IgM $F(ab')_2$ fragments (Jackson ImmunoResearch, West Grove, PA) were added and calcium influx was measured for 5 min. Subsequently, 2 μg/mL ionomycin (Calbiochem, San Diego, CA) was added. For further studies, cells were resuspended in Ca^{2+}-free phosphate buffered saline (PBS) with 10% FCS. To define the role of CD20, PBMC were incubated with 10 μg/mL of the anti-CD20 mAb rituximab[25] (Roche Diagnostics, Mannheim, Germany) for 30 min. Epstein-Barr virus (EBV) transformed lymphoblastoid B cell lines (LCL) were generated.[26] ER Ca^{2+} storage capacity was assessed with ionomycin (0.1μg/mL) or thapsigargin (10 μM, Santa Cruz Biotechnology, Santa Cruz, CA) in Ca^{2+}-free medium.

B lymphocyte differentiation assays

Assays were performed as described previously.[23] Briefly, PBMC were cultured with *Staphylococcus aureus* Cowan I strain antigen (40 U/mL) and IL-2 (10 U/mL, Sanquin). After 7 days, cells were harvested, cytospins were prepared and stained with FITC-conjugated anti-IgM, IgA or IgG (Southern Biotechnology Associates). Ten fields of 50

cells were analyzed by fluorescence microscopy and the fraction of plasmablasts was calculated.

Confocal microscopy

Control and CVID disorder LCL were sorted for high expression of IgM and CD20. Two million B cells were adhered on a coverslip coated with 1% alcian blue (Klinipath bv, Geel, Belgium). Fc receptors were blocked and cells were kept at 0°C to stain the IgM BCR and CD20 with 1:100 goat anti-human IgM-Cy3 $F(ab')_2$ fragments (Jackson ImmunoResearch) and 1:10 anti-human 2H7-Alexa Fluor 647 (BioLegend, San Diego, CA), respectively for 45 min. Cells were washed 3 times and resuspended in 1640 RPMI without phenol red, containing 10% FCS and 10 mM HEPES buffer. Cells were kept at 0°C and imaged on a 710 Laser Scanning Microscope Meta (Carl Zeiss MicroImaging GmbH, Germany). BCR stimulation was initiated by quickly increasing the temperature to 37°C and supplementing additional anti-IgM. Temperature was kept on 37°C. Imaging was continued for 30 min. Using ZEN 2009 Software, the percentage of colocalization was calculated as described previously.[22]

Statistical analysis

Data are shown as scatterplots with median, or bar graphs with mean ± standard error of the mean. Differences in nonparametric data were assessed by comparing the medians of groups using Mann-Whitney *U* tests or Kruskal-Wallis tests (multiple groups), followed by post hoc Dunn's comparisons. The effect of αCD20 treatment was assessed with paired t-tests and correlations were calculated with Pearson correlation. All tests were performed two-tailed and $p \leq 0.05$ was considered significant. Data were analyzed using SPSS 15.0 for Windows (SPSS Inc.; Chicago, IL). Areas under curve were calculated using Graphpad Prism 5.0 software (La Jolla, CA).

RESULTS

Pediatric CVID disorder B cells are reduced in memory subsets but display a normal surface phenotype

Study of the B cell compartment demonstrated that CVID and CVID-like children had lower percentages of memory cells than both control groups: $CD27^+IgM^-$ Ig class-switched memory B cells, median ±interquartile range, healthy adults 14.6% ±15.8; healthy children 7.1% ±5.3; CVID disorders 4.4% ±4.5, p=0.01; $CD27^+IgM^+$ memory cells, healthy adults 25.5% ±23.4; healthy children 11.2% ±13.3; CVID disorders 7.9% ±9.1, p=0.001. No differences were observed in $CD38^-CD21^{low}$ B cell populations between CVID patients and controls, although in two patients with active autoimmune disease we did notice expansion of $CD21^{low}$ cells *(figure S1, A of the Supplementary Figures)*. To determine if CVID B cells exhibit a deficiency in BCR-mediated signaling due to altered surface expression of BCR co-receptors, we measured surface CD19, IgM BCR, CD20 and CD22. CD19 and IgM expression on individual B cell subpopulations

were comparable between patients and controls (*figure S2, A*); IgM expression was slightly increased on $CD27^+IgM^+$ B cells in CVID disorders compared to healthy children (MFI*10^3 healthy adults 11.6; healthy children 10.6 ±6.6; CVID disorders 13.5 ±5.8, p=0.03) (*figure S2, B*). We observed no significant differences in CD20 and CD22 expression (*figure S2, C and D*). In line with previous studies, we found no significant differences between CVID and CVID-like patients.[23] In conclusion, all subjects had a substantial IgM^+ B cell population that displayed a normal surface phenotype.

Impaired BCR crosslinking-induced calcium mobilization and defective B cell differentiation in vitro in CVID disorder B cells

We analyzed the ability of fresh CVID and CVID-like B cells to mobilize calcium upon B cell activation. Baseline calcium levels of B cells were similar in all groups. Upon αIgM $F(ab')_2$-mediated BCR cross-linking, B cells from both CVID and CVID-like children exhibited reduced calcium mobilization, as measured by area under curve (AUC) (*figure 1, A*: AUC median ±interquartile range, healthy adults 200 ±221; healthy children 263 ±120; CVID 124 ±95; CVID-like 93 ±69, p<0.001). The initial peak calcium influx was mainly decreased, while further kinetics patterns appeared unaffected. Calcium ratios (peak/baseline)[12] were indeed also significantly decreased compared to controls (calcium ratio healthy adults 2.9 ±1.5; healthy children 2.9 ±0.8; CVID 2.2 ±0.6; CVID-like 1.9 ±0.8, p<0.001). We could not classify patients with a decreased calcium flux into certain subgroups, but noticed that patients with symptoms of autoimmunity or lymphoproliferation had relatively high calcium mobilization (*figure 1, A and S1, B*). Since there were no differences between CVID and CVID-like patients, those patient groups were combined in further experiments and further addressed to as 'CVID disorders'.

Restimulation of αIgM $F(ab')_2$-treated B cells with ionomycin, which acts directly on IP_3 receptors at the ER membrane, induced sub-optimal subsequent calcium mobilization in CVID $CD19^+$ B cells (calcium ratio healthy adults 17.2 ±4.8; healthy children 19.4 ±6.6; CVID 11.5 ±5.5; CVID-like 10.4 ±10.0, p<0.001). In contrast, $CD19^-$ CVID disorder lymphocytes (mostly T cells) showed fluxes comparable to control $CD19^-$ lymphocytes (data not shown).

We next asked whether decreased calcium mobilization would lead to subsequent B cell defects. BCR-mediated calcium signals correlated significantly with *in vitro* differentiation into plasmablasts upon stimulation with IL-2 and *Staphylococcus Aureus* Cowan strain I antigen, which binds the BCR, and elicits a T-independent B cell response,[27-29](*figure 1, B*,) and with numbers of $CD19^+CD27^+IgG^+$ memory B cells. (*figure 1, C and table SI*) BCR-mediated upregulation of costimulatory molecules and nuclear factor of activated T cells (NFAT) gene transcription was comparable between patients and controls (*figure S3*). Thus, CVID B cells exhibit a selective deficit in BCR-mediated calcium mobilization, which positively correlates with decreased plasmablast differentiation *in vitro* and class-switched memory B cell formation or maintenance *in vivo*, at least under our experimental conditions.

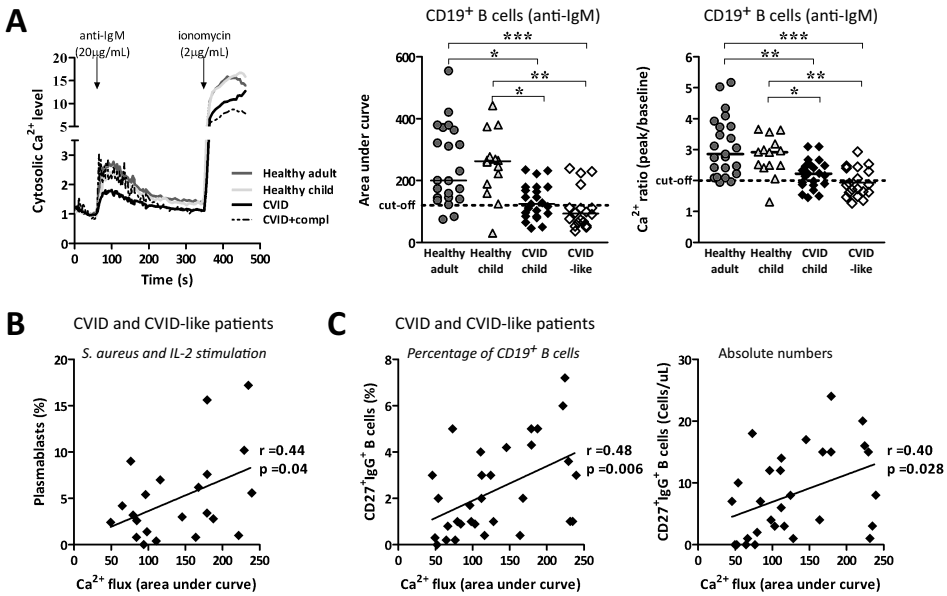

Figure 1: Decreased calcium mobilization in CVID disorders B lymphocytes. A, Ca^{2+} mobilization kinetics (i), area under curve (ii) and ratios (iii). Cut-off levels are at a level above which 90% of the healthy controls are positive. B, B cell differentiation correlated with Ca^{2+} mobilization in CVID disorders. C, Percentages (i) and absolute (ii) numbers of IgG^+ memory B cells correlated with BCR-mediated calcium influx in age-corrected CVID disorders (n=30). Pearson's correlations. * p<0.05; ** p<0.01; *** p<0.001.

Defective calcium mobilization is not caused by defects in plasma membrane channels, ER calcium storage release, and BCR signaling molecules Btk or PLCγ2

The function of plasma membrane calcium channels was evaluated using calcium-free assays. Despite lacking exogenous calcium influx, AUC and calcium ratios remained significantly lower in CVID disorders compared to controls (*figure 2, A*: AUC controls 276 ±124; CVID disorders 83 ±223, p=0.02). Additionally, calcium-depleted LCL generated from CVID patients showed similar transmembrane calcium influx upon $CaCl_2$ supplementation as did control LCL (*figure 2, B:* AUC control 37 ±19; CVID disorders 47 ±53, p=0.3).

To investigate ER calcium release, control and patient LCL were treated with ionomycin or thapsigargin (*figure S4*). Thapsigargin inhibits SERCA pumps, which maintain ER calcium stores.[30] Both treatments led to a similar pattern of calcium release into the cytosol between patient and control LCL. To survey the function of BCR signaling cascade molecules, relevant tyrosine phosphorylation sites of *BTK*[31] and *PLCG2*[32;33] were sequenced in sixteen patients exhibiting reduced calcium ratios, but no mutations were found (*Supplementary Methods*).

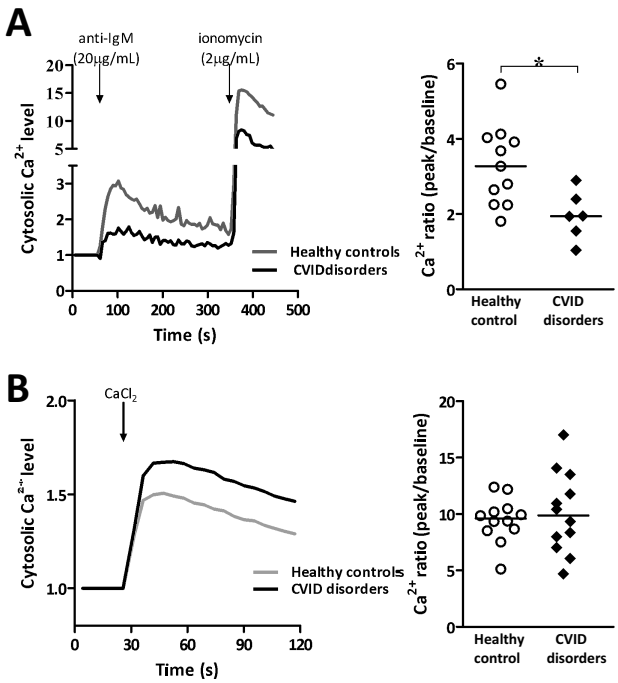

Figure 2: Plasma membrane calcium channels function in CVID disorders B cells. A, ER Ca^{2+} release upon anti-IgM in calcium-depleted medium; averaged kinetics plot (i) and Ca^{2+} ratios (ii) in B cells of 11 controls and 6 CVID disorders patients. B, $CaCl_2$ addition induces Ca^{2+} influx in patient and control LCL (both n=12). Lines indicate medians. Mann-Whitney *U* test, * $p<0.05$.

Taken together, protein function of Btk and PLCγ2, as well as ER calcium homeostasis and plasma membrane channel function appeared intact in our CVID disorder B cells. Henceforth, we focused on immediate BCR activation events in the plasma membrane.

Contribution of CD20 to activation-induced calcium mobilization defect in CVID disorder B cells

BCR and CD20 constitutively partly colocalize at the B cell plasma membrane (*figure 3, A and* [22]). CD20 mAb treatment specifically increased cytosolic calcium levels in healthy and CVID disorders B cells after 30 minutes (*figure 3, B*). Additionally, CD20-specific mAb ligation of CD20 caused internalization of the BCR/co-receptor complex, as noticed by significant reduction in surface expression of Igλ light chains, CD21 and CD19, but not CD45, at 37°C but not at 4°C (*figure 3, C* 37°C, and data not shown).

We next asked whether defective lateral dissociation of BCR from CD20 could explain defective calcium mobilization in CVID disorder B cells. B lymphocytes were pre-treated with CD20-specific mAb for 30 min to induce CD20 crosslinking-induced B cell activation, as shown previously and in figure 4B.[20;34] The effectiveness in BCR/CD20 dissociation was measured by inducing a secondary calcium flux upon BCR stimulation. We found that healthy but not patient B cells were hyporesponsive to subsequent BCR triggering, reflecting that initial elicitation of the B-signaling pathway by the anti-CD20 treatment was productive in healthy but not CVID disorder B cells (*figure 4, A*: healthy control calcium ratio only αIgM 2.2 ±1.1; with αCD20+αIgM

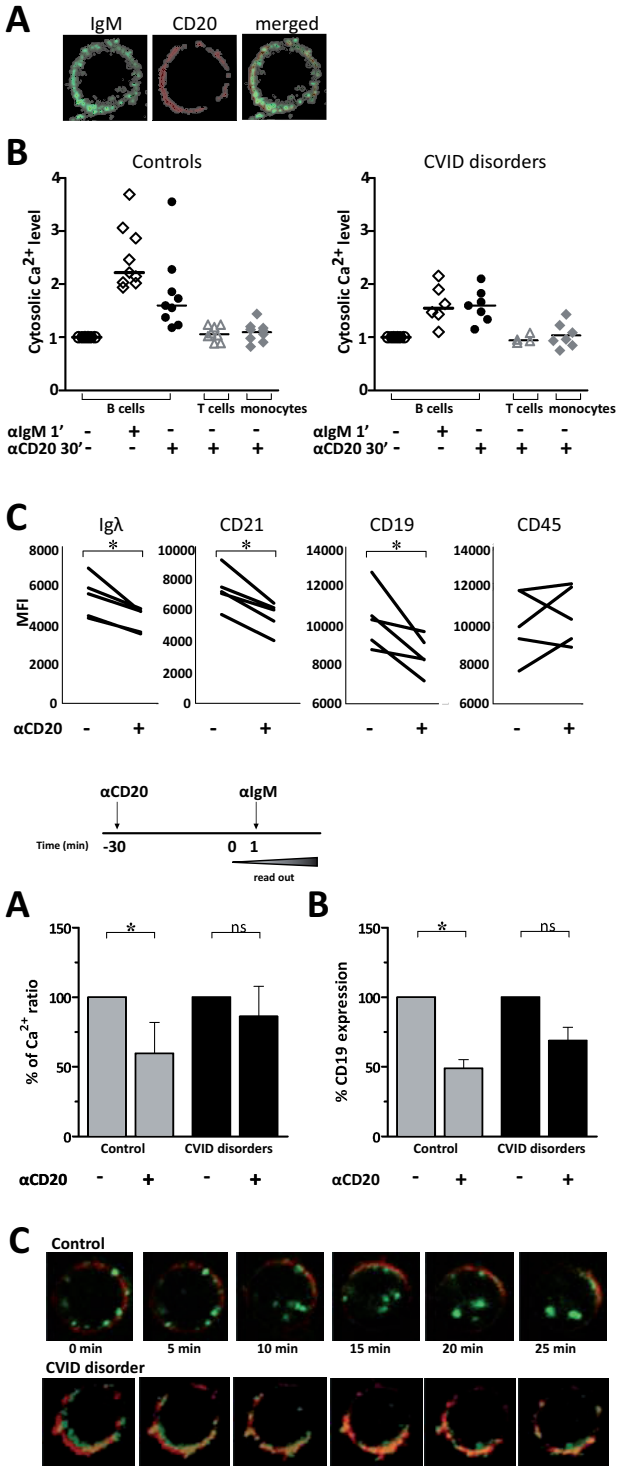

Figure 3: Anti-CD20 induces calcium influx and BCR/co-receptor internalization. **A,** Colocalization of IgM *(green)* and CD20 *(red)* in resting B cells. **B,** Anti-CD20 triggers a specific Ca^{2+} flux in healthy (i) and CVID disorder (ii) B lymphocytes. **C,** Anti-CD20 treatment of healthy B cells leads to selective internalization of molecules clustered in the BCR/co-receptor complex. Paired t-test, * $p<0.05$.

Figure 4: Anti-CD20 does not exhaust BCR-mediated calcium mobilization in CVID disorders. **A-B,** Anti-CD20 pre-treatment decreases Ca^{2+} mobilization *(A)* and CD19 surface expression *(B)* in primary B cells of 10 controls and 8 patients. Mean ± SEM. Paired t-test, * $p<0.05$ **C,** IgM BCR *(green)* and CD20 *(red)* dissociate upon anti-IgM $F(ab')_2$ stimulation of control and CVID disorder LCL.

1.3 ±0.35, p=0.004; CVID disorders only αIgM 1.8 ±0.47; αCD20+αIgM 1.4 ±0.61, p=0.16). Direct triggering of CD20 exhausted subsequent receptor-mediated calcium fluxes in healthy but not patient B lymphocytes. Further, CD20-mAb treatment significantly decreased surface expression of CD19 surface molecules in healthy controls (CD19 MFI*10^3 without αCD20 4.78 ±1.65, with αCD20 2.00 ±1.07, p<0.001), but not in patients (without αCD20 2.87 ±1.24, with αCD20 1.68 ±0.56, p=0.08) (*figure 4*, *B*).

We hypothesized that the development of CVID disorders may involve defects in the activation-induced disassembly of CD20 from the BCR signalosome,[19] and visualized CD20 and BCR plasma membrane mobility upon BCR crosslinking on healthy control and CVID disorder IgM^+ LCL cells. Upon BCR-crosslinking, dissociation and subsequent internalization of the BCR was observed, while CD20 remained at the cell surface.[19;22] This process seemed less effective in the CVID disorder cells (*figure 4, C and figure S5*).

In conclusion, we show that calcium signaling is disturbed in CVID disorder B lymphocytes, and can be contributed to defective dissociation of the BCR from CD20 at the plasma membrane. Our findings suggest defective kinetics of the disassembly of BCR signalosome oligomers to be a contributing factor in the development of CVID disorders in children.

DISCUSSION

We describe a selective defect in calcium mobilization in B lymphocytes observed in numerous pediatric CVID disorder patients, which directly correlates with abrogated plasmablast development *in vitro* and decreased numbers of class-switched memory B cells *in vivo*. We show that CD20 dissociation from the BCR/co-receptor complex on B cells is required for activation-induced calcium signaling and propose that failing of this process may contribute to development of CVID disorders.

Calcium fluxes are pivotal for lymphocyte function, and cytosolic calcium levels are tightly regulated. Calcium signaling influences cell-fate choices by differential regulation of transcription factor pathways including NFAT and nuclear factor kappa B (NFκB).[35] We found that decreased calcium mobilization correlated with decreased B cell differentiation into plasmablasts upon BCR stimulation, and with decreased fractions of IgG^+ memory B cells in peripheral blood, suggesting an important role for BCR-mediated calcium mobilization in the development or maintenance of these memory cells *in vivo*. Defective B cell activation could thus explain the B cell abnormalities characteristic of CVID disorders. B cell calcium mobilization was defective in a larger proportion of our pediatric CVID disorder cohort than was previously described in an adult CVID population, and we did not find an increased expression of the inhibitory B cell co-receptor CD22.[12] Calcium fluxes were measured in fresh unfractionated $CD19^+$ B cells. We asked whether decreased calcium fluxes in CVID patients could be explained by differences in composition of the B cell compartment but confirmed that CVID patients have a relatively larger IgM^+ B cell compartment than healthy adults and

children.[23;24] Moreover, calcium mobilization was reduced in all post-transitional B cell subpopulations in adult CVID,[12] and our decreased Ca^{2+} ratios did not correlate with percentages of IgM^+ B cells (our unpublished observations).

In this study cohort, we tested the capacity of plasma membrane calcium channels and ER calcium storage capacity upon cellular activation, and found them in CVID B cells to be comparable to healthy controls. These features are conserved between lymphocytes and defects would likely cause a more severe, combined immunodeficiency.[36;37] Nevertheless, we cannot completely exclude the possibility that sporadic CVID disorder patients have smaller defects in less pertinent or yet unidentified channels, or in BCR-specific phosphotyrosine kinases. As key residues of *PLCG2* and *BTK* were not mutated in calcium signaling-affected cells, it is unlikely that these will be etiologic factors in a considerable proportion of the CVID population. Complete dysfunction of such molecules usually leads to negligent numbers of peripheral B cells and agammaglobulinemia,[38-40] which was not seen in this cohort.

During B cell activation, the plasma membrane undergoes dynamic remodeling.[22;41] We hypothesized this remodeling to be defective. CD20 homo-oligomers associate with the BCR in lipid microdomains in resting cells.[22;34;42] BCR stimulation causes dissociation of the BCR from CD20, with BCR persisting in lipid microdomains.[19] Direct triggering of CD20 by anti-CD20 mAb (rituximab) causes an induction of calcium flux, which requires association of CD20 with the BCR, and is mediated via the BCR signaling.[20;21] CD20 ligation on healthy B cells triggered a calcium flux that exhausted the BCR signaling cascade, as subsequent BCR-triggering scarcely induced subsequent calcium signaling. These findings support recent work, describing that αCD20 pre-treatment results in a time-dependent inhibition of the BCR signaling cascade in healthy human B cells.[25] In contrast, in CVID disorder B cells, we show that αCD20 pre-treatment triggers an initial calcium flux, but does not yield complete BCR-mediated signaling, as further residual calcium signaling can be elicited when BCRs are restimulated after 30 min (*figure 4*).

We propose that upon CD20 and/or BCR triggering, BCR-CD20 signalosomes induce downstream signaling as described above and elsewhere, followed by rapid dissociation at the cell surface, where CD20 remains while the BCR/co-receptor complex is endocytosed,[19;22] and triggering calcium signaling in the cytosol.[20;25] We first stimulated with anti-CD20 and subsequently added anti-IgM. We chose this order, as anti-CD20 treatment requires more time than anti-IgM to elicit calcium fluxes, as measured by in real time by flow cytometry. However, as both antibodies trigger the same signaling route, either order of (re)stimulation should yield similar results. Our data suggests this synergistic mechanism to be impaired in a subgroup of CVID disorder patients, rendering B cells less capable to resolve CD20/BCR oligomers, resulting in reduced BCR/co-receptor internalization and decreased calcium signaling. It remains to be identified which genes are involved in this process.

In conclusion, we found a calcium mobilization defect in B lymphocytes of children with CVID disorders, which we propose results from altered dissociation of the BCR from CD20. Other additional signalosome-associated proteins may be involved as well. Further studies are required to address the molecules involved in CD20-

associated BCR-receptor signaling, the underlying genetic defects leading to the CVID disorder phenotypes, for elucidation of possible intervention points for B cell activation targeted therapy.

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SUPPLEMENTARY METHODS

Patients

Of the 42 patients, 23 were diagnosed with CVID according to the ESID criteria.[S1] All but two newly diagnosed patients received immunoglobulin replacement therapy. Nineteen did not completely meet these CVID criteria and were therefore diagnosed with 'CVID-like' disease; defined as selective antibody deficiency (defined as the inability to produce protective titers of specific antibodies upon vaccination to polysaccharide and/or to recall antigens), combined with low IgA, low IgM, and/or low IgG subclass levels, or a solitary decreased IgG. These patients had recurrent infections and an inadequate response to prophylactic antibiotic treatment, which was defined by more than 4 breakthrough infections per year. Secondary immunodeficiencies (e.g. iatrogenic or due to enteral protein loss) were ruled out in all patients. Previous studies have shown that pediatric CVID-like patients as described above are clinically and immunologically (e.g. B and T cell phenotype characterization) comparable to definite CVID patients.[S2;S3] As the current flow cytometry-based CVID classifications are not applicable to children,[S2] we did not attempt to classify them. Five children had however already developed disease-related complications as described by Chapel *et al.*[S4] These were enteropathy, autoimmune hemolytic anemia (3x), idiopathic thrombocytopenic purpura (3x), autoimmune nephritis, hepatosplenomegaly with extensive lymphadenopathy, lymphoid interstitial pneumonia, systemic lupus erythematosus and vitiligo.

Ten healthy pediatric controls were asked to donate blood when elective surgery was performed. Leftover peripheral blood (1 mL) was used from four healthy pediatric controls of another approved study in our lab. All adult volunteers were healthy employees of the University Medical Center Utrecht.

Genetic analysis of *PLCG2* and *BTK*

Granulocytes were isolated from peripheral blood with the use of Ficoll gradient centrifugation and subsequently genomic DNA was isolated using the autopure kit (Qiagen). Exon 22 of the *PLCG2* gene (NM_002661.2) and exon 18 of the *BTK* gene (NM_000061.2) were directly sequenced after PCR on an ABI 3100 automated sequencer (PE Applied Biosystems, Foster City, CA, USA) and a 3130XL genetic analyzer using the Applied Biosystems BigDye Terminator v1.1 cycle sequencing kit

according to the manufacturer's protocol. Analysis was done using the SeqScape v2.5 (Applied Biosystems) and Mutation Surveyor software (Softgenetics, LLC, State College, PA, USA). PCR conditions and primer sequences are available upon request.

B cell cultures

PBMC were thawed quickly, washed twice and resuspended in RPMI (Gibco) containing 10% fetal calf serum, 1% penicillin and streptavidin and 1% glutamine in a 24-well plate for 24 or 48 hours in with following stimuli: PMA (50ng/mL) and ionomycin (2ug/mL, Calbiochem, San Diego, CA) as a positive control, anti-IgM $F(ab')_2$ fragments (20 ug/mL, Jackson Immunoresearch, West Grove, PA) and anti-IgM $F(ab')_2$ fragments (10ug/mL) with IL-4 (200 U/mL, Immunotools, Friesoythe, Germany). After 24 hours, cold FACS buffer was added and cells were washed twice. After Fc receptor blocking with mouse serum, cells were stained for CD19, CD80 and CD86 (all Becton Dickinson) for 30 minutes at 4 °C. After washing twice, cells were acquired on FACS Canto II Flow Cytometer and analyzed using FACS Diva software (both Becton Dickinson). After 48 hours, cells were lysed and total mRNA was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and reverse using an iScript cDNA synthesis kit (Biorad, Hercules, CA). Primers were mixed with IQ SYBR green supermix (BioRad). The detection run started at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Assays were performed in triplicate on a C1000 Thermal Cycler (BioRad). Results were normalized to endogenous GAPDH mRNA and the unstimulated sample ($2^{-\Delta\Delta CT}$). The following primers were used: GAPDH Forward: 5'-GTCGGAGTCAACGGATT-3'; GAPDH Reverse: 5'-AAGCTTCCCGTTCTCAG-3'; NFAT Forward: 5'-GCAGAGCACGGACAGCTATC-3'; NFAT Reverse 5'-GGGCTTTCTCCACGAAAATGA-3' (All Sigma-Aldrich).

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Table S1: Distribution of B cell subpopulations in patients with normal and decreased calcium mobilization.

	CVID disorder patients (n =33, aged 7-18)			
	Normal calcium mobilization (n=16, 49%)		Decreased calcium mobilization (n=17, 51%)	
B cell subpopulations	**% Total B cells**	**Numbers (cells/μL)**	**% Total B cells**	**Numbers (cells/μL)**
$CD19^+$ B cells, *median (interquartile range)*	N/A	338 (217)	N/A	308 (272)
$CD19^+IgD^+CD10^+CD38^+$ recent bone-marrow emigrants	12 (12)	40 (41)	15 (13)	55 (45)
$CD19^+IgD^+IgM^+CD10^-CD27^-$ naive B cells	64 (23)	215 (218)	69 (16)	222 (641)
$CD19^+IgD^+IgM^+CD27^+$ 'memory IgM' B cells	6.0 (5.1)	17 (55)	2.5 (5.3)*	11 (24)
$CD19^+IgG^+CD27^+$ memory IgG B cells	3.3 (4.0)	15 (13)	1.0 (2.2)*	4 (11)*
$CD19^+IgA^+CD27^+$ memory IgA B cells	1.5 (1.3)	5 (7)	1.0 (1.3)	2 (5)

N/A=not assessed; * p<0.05, Mann-Whitney *U* test

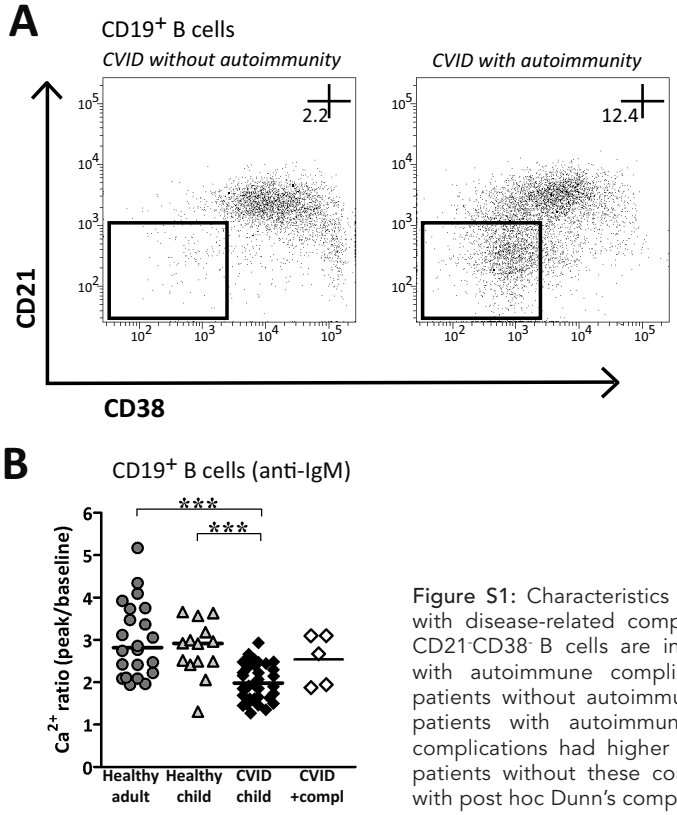


Figure S1: Characteristics of CVID disorder patients with disease-related complications. A, Autoreactive $CD21^-CD38^-$ B cells are increased in CVID patients with autoimmune complications *(ii)* compared to patients without autoimmunity *(i)*. B, CVID disorder patients with autoimmune or lymphoproliferative complications had higher calcium mobilization than patients without these complications. Kruskal-Wallis with post hoc Dunn's comparisons, *** p<0.001.

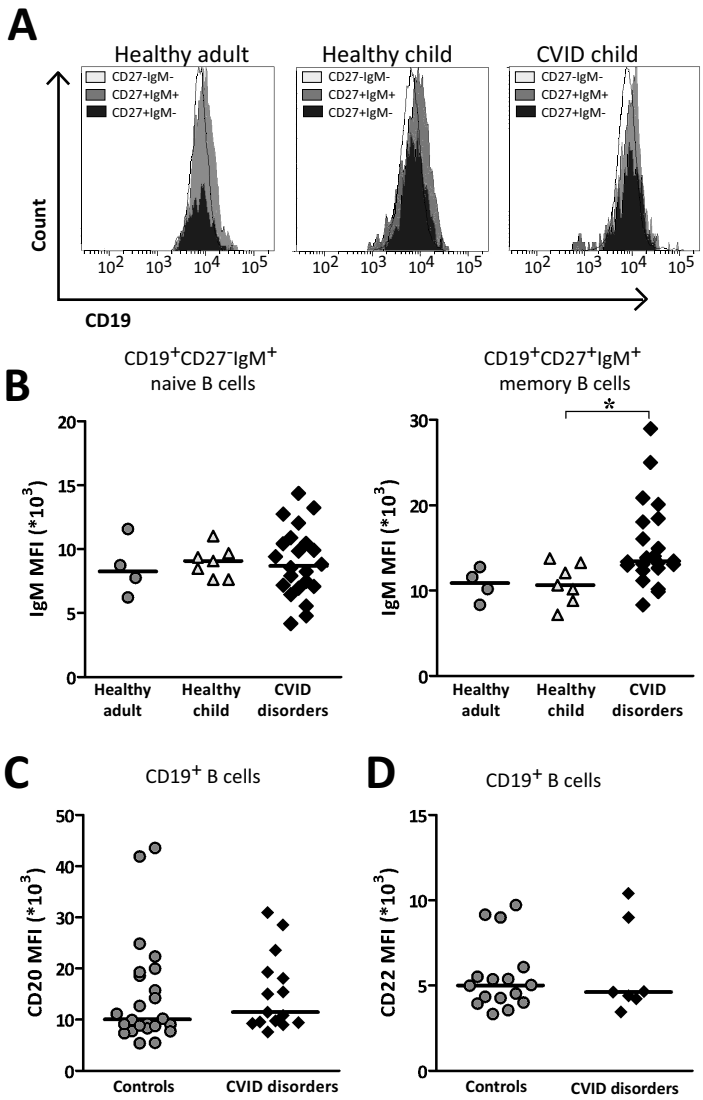


Figure S2: Expression of B cell surface molecules in CVID disorders. A, CD19 expression in 5 adults, 10 healthy children and 34 CVID disorder children. B, Mean fluorescence intensity (MFI) of IgM on naive B cells and memory B cells. C and D, CD20 (*C*) and CD22 (*D*) expression in patients and controls. Kruskal-Wallis with post hoc Dunn's comparisons, * $p<0.05$.

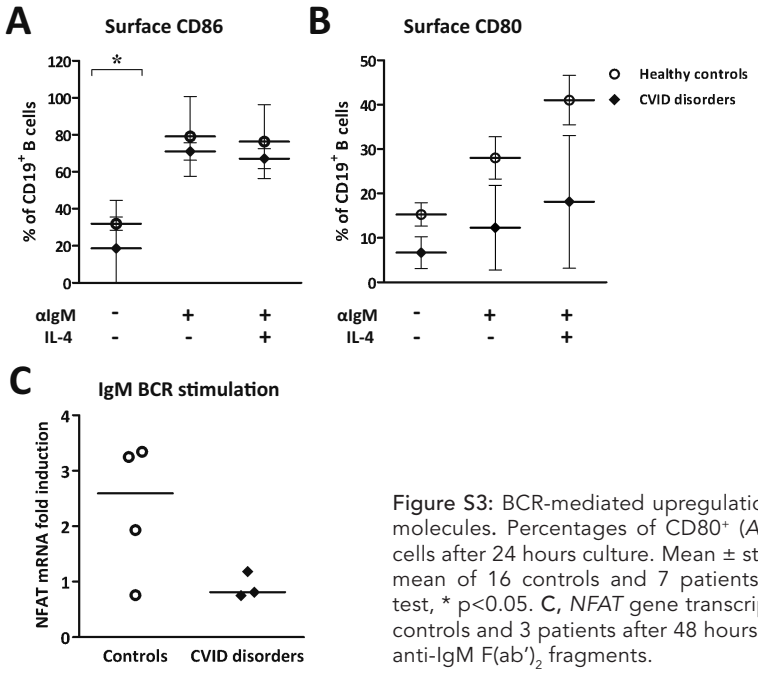


Figure S3: BCR-mediated upregulation of costimulatory molecules. Percentages of $CD80^+$ (*A*) and $CD86^+$ (*B*) B cells after 24 hours culture. Mean ± standard error of the mean of 16 controls and 7 patients. Mann-Whitney U test, * $p<0.05$. C, *NFAT* gene transcription in PBMC of 4 controls and 3 patients after 48 hours of stimulation with anti-IgM $F(ab')_2$ fragments.

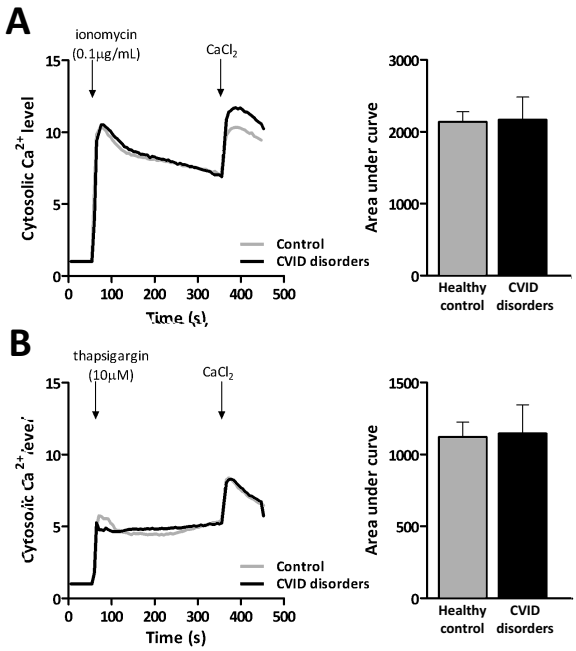
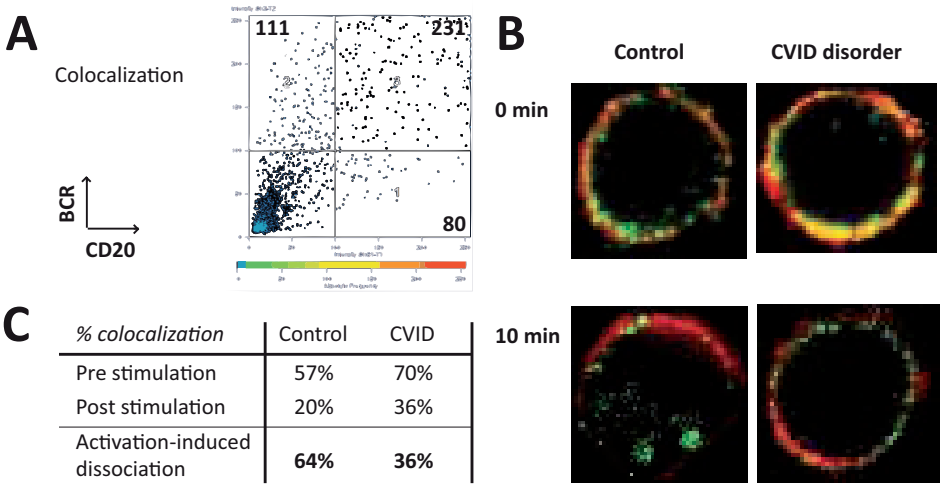


Figure S4: ER calcium mobilization and storage capacity in CVID disorder B lymphocytes. Kinetics of ER storage Ca^{2+} release after ionomycin stimulation *(A)* or thapsigargin depletion *(B)* in calcium-depleted medium, demonstrated by an averaged kinetics plot *(i)* and area under curve *(ii)* in 6 control and 6 CVID disorder LCLs. Bar graphs represent mean ± standard error of the mean.



C

% colocalization	Control	CVID
Pre stimulation	57%	70%
Post stimulation	20%	36%
Activation-induced dissociation	**64%**	**36%**

Figure S5: Impaired BCR-induced BCR-CD20 lateral dissociation in CVID disorders. **A,** Colocalization was calculated by dividing double positive pixels (BCR/CD20) by the positive pixels within the BCR image. **B,** Control *(i)* and CVID disorder *(ii)* LCL before *(upper panel)* and after BCR stimulation *(lower panel)*. **C,** Percentage of dissociation upon BCR triggering, based on >25 cells per donor in two experiments.

6

DISTINCT IMMUNOLOGICAL FEATURES IN SIBLINGS SHARING AN A181E/C104R COMPOUND HETEROZYGOUS MUTATION IN TRANSMEMBRANE ACTIVATOR AND CALCIUM-MODULATING CYCLOPHILIN LIGAND INTERACTOR (TACI)

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ABSTRACT

Common variable immunodeficiency, CVID, is a primary antibody deficiency for which etiology has remained largely unknown. Approximately 9% of patients harbor variants of the transmembrane activator and CAML interactor gene, TACI, contributing to CVID development.

We found identical compound heterozygous TACI variants (C104R and A181E) in kindred of which one individual had CVID, one sibling developed the disease during follow-up and of which a second sibling remained asymptomatic. Despite having an identical TACI genotype, intracellular and B cell surface expression levels of TACI were higher in the asymptomatic sibling than the CVID patient, as well as TACI-mediated gene transcription of activation-induced deaminase (AID) and nuclear factor of activated T cells (NFAT). In analogy, the asymptomatic sibling displayed enhanced Toll-like receptor (TLR) 9 expression and signaling, suggesting a compensatory immune mechanism, as TACI and TLR9 share their signaling pathway. These findings suggest that post-transcriptional regulation of TACI protein and cross-talk with TLR9 signaling may contribute to the observed phenotypic diversity between individuals with TACI variants.

INTRODUCTION

Common variable immunodeficiency (CVID) is a primary immunodeficiency characterized by recurrent bacterial infections, hypogammaglobulinemia and impaired antigen-specific antibody synthesis.[1] In around 10% of patients, a genetic defect has been identified. In addition, functional defects have been described, e.g. in Toll-like receptor (TLR) signaling.[2;3] The most prevalent genetic alterations are located in the *TNFRSF13B* gene encoding transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor (TACI).[4] TACI is mainly expressed on B cells and belongs to the tumor necrosis factor receptor (TNFR) family.[4] Two ligands have been described: B cell activating factor (BAFF,[5] also described as BLyS,[6] zTNF4,[7] TALL-1,[8] THANK[9]) and a proliferation-inducing ligand (APRIL);[10;11] in addition, TACI may bind proteoglycans including syndecan-2.[12;13]

BAFF and APRIL also bind B cell maturation antigen (BCMA)[14] and BAFF has a third receptor, BAFFR.[15] Due to this versatility, the contribution of each molecule to humoral immunity is not yet delineated.[13]

Following ligand binding, the intracellular TACI domains interact with TNFR-associated proteins (TRAFs) and CAML. CAML interaction activates nuclear factor of activated T cells (NFAT), which has a key role in controlling calcium release from intracellular stores.[4] Additionally, nuclear factor kappa B (NFκB) is activated,[16] probably via TRAFs, and c-Jun NH2-terminal kinase and transcription factor AP-1.[4] TACI has a role in maintenance of B cell homeostasis, immunoglobulin (Ig) isotype class-switching and antibody production in response to type II T cell-independent antigens. Furthermore, TACI can act in synergy with CD40 and TLRs;[17-19] it was recently shown that TACI induces class-switch recombination via the myeloid differentiation primary response gene 88 (MyD88) adaptor, thus employing the same signaling cascade as most TLRs.[20]

In 2005, two papers described homozygous and heterozygous mutations in patients with CVID or IgA deficiency, and in none of 50 healthy controls.[21;22] Two years later however, further screening revealed that heterozygous variants were present in the healthy population.[23;24] Currently, TACI is considered a susceptibility gene, of which (heterozygous) disease-associated variants are present in ~9% of the CVID patients, compared to only monoallelic variants in <2% of the healthy controls.[25] In healthy or mildly affected family members of CVID patients, some biallelic polymorphisms were found, suggesting that TACI variants may have an incomplete penetrance.[25] Furthermore, the presence of TACI polymorphisms in CVID predisposes to autoimmune disease and lymphoid hyperplasia.[26]

Two TACI variants are significantly associated with CVID disease: C104R[24;25] and A181E.[24] A characteristic of TNFRs is the presence of two conserved cystein-rich domain (CRDs) in the extracellular domain.[27] TACI monomers engage in a ligand-independent homotypic trimer formation that is dependent on the CRD1 domain;[28] the second CRD allows ligation.[29] The amino acid substituting C104R variant is located in CRD2 and thus abrogates ligand binding,[21;22] as was demonstrated in CVID patients heterozygous for C104R. Initially, the deficiency was attributed to a dominant-negative

interference of the C104R polymorphism in trimeric complexes with TACI wildtype;[28] subsequent reports however suggest haploinsufficiency of the TACI allele.[30-32]

The A181E variant is located in the transmembrane region and does not interfere with ligand binding.[21] Mouse models with a homozygous A181E equivalent (A144E) show defective pre-ligand and ligand-induced clustering of the intracellular domain and subsequent hampered NFκB activation; possibly, this is caused by a conformational change resulting from the negative charge introduced by the A181E variant.[33] A further consequence was low serum IgA levels and impaired antibody responses to type II T cell-independent antigens.

To more closely resemble human conditions exhibiting heterozygous alleles, A181E and C104R were investigated in combination with a wildtype TACI allele. Neither variant interfered with oligomerization with wildtype TACI molecules, and NFκB activation was unaffected in 293T cells transfected with wildtype and mutant TACI.[31] Dominant-negative interference of the variants with wildtype TACI is therefore improbable, but haploinsufficiency has not been excluded completely. It was therefore recommended that B cell function is studied in individuals who carry the same mutation (both patients, their healthy relatives and unrelated subjects).[31]

We identified a CVID patient compound heterozygous for A181E and C104R. Her disease course was complicated by autoimmune nephritis and cytopenias.[34] In contrast, her two siblings with identical TACI genotype were healthy, which has not been reported previously. Here we describe a clinical and laboratory evaluation of the patient in comparison to the healthy sibling and unrelated healthy and CVID controls. The A181E/C104R heterozygous mutation in the patient is associated with decreased AID and NFAT expression, while the healthy sibling's B cells showed normal AID and NFAT expression. The healthy sibling showed increased TACI and TLR9 expression, which we propose may offset the A181E/C104R predisposition to CVID. Thus, we provide mechanistic support how individuals with identical TACI alleles may exhibit differential TACI protein expression and downstream signaling function.

MATERIALS AND METHODS

Case and family

The index patient (*figure 1, A,* arrow) presented at age 11 with recurrent upper and lower respiratory tract infections and splenomegaly. Laboratory investigations showed absent isohemagglutinins, decreased serum titers of total IgG, IgA, IgG_1 and IgG_2 *(table 2)*. She was lymphopenic, with low numbers of B cells, $CD4^+$ and $CD8^+$ T cells. Memory B cells were decreased (class-switched memory B cells <2% of total B cells). Functionally, *in vitro* T cell proliferation assays showed a positive response to mitogens and an incomplete response to particularly viral antigens. A diagnosis of CVID was made and immunoglobulin replacement therapy was initiated. The index patient however developed CVID-related disease manifestations, including autoimmune hemolytic anemia (AIHA) and idiopathic thrombocytopenic purpura (ITP) (hemoglobin 5.4mmol/L and platelet count $38*10^9$/L), submandibular lymphadenopathy and autoimmune

nephritis (renal biopsy showing tubulo interstitial nephritis with granulomatous changes; estimated glomerular filtrated rate, GFR, calculated by Schwartz formula was 41.2 ml/min/1.73m^2). Furthermore, she developed exercise-induced dyspnea; a high-resolution computed tomography (HRCT) scan showed airway and interstitial lung disease with bronchiectasis and peribronchovascular abnormalities (*figure 1*, *B*). A broncho-alveolar lavage showed no indication for viral, bacterial or fungal infections. Pulmonary findings were suggestive for granulomatous disease, and low-dose steroids and tacrolimus were initiated, resulting in normalization of pulmonary function and of GFR. Furthermore, viral immunity appeared to be affected, as cytomegalovirus was repeatedly detectable (viral loads 100-2000 copies/mL) in plasma. Treatment with tacrolimus eventually resulted in tubulopathy and mycophenolate mofetil was started with good initial response, however causing pancreatitis. Rituximab had a positive effect on the lymphadenopathy, AIHA and ITP. As part of our routine evaluation of CVID patients, C104R and A181E TACI variants were screened by polymerase chain reaction (PCR) as described previously,[34] and both were found in the index patient. As we considered stem cell transplantation in the index patient, her parents and siblings (*figure 1, A*) were also screened. Informed consent was obtained to further evaluate the patient and family members for clinical indications and research purposes. Both parents and the two sisters were vaccinated with 23-valent Pneumovax (Adventis Pasteur MSD) and specific antibodies were measured after 1 month. Further data were obtained from the medical records.

Flow cytometry

Phenotypic and functional evaluation of the lymphocyte compartment was performed as described previously.[34;35] Next, lymphoblastoid cell lines (LCL) were generated using Epstein-Barr virus and stained with antibodies against CD19, CD20, CD21 (all Becton Dickinson), TLR9, CD40 (both eBioscience), rabbit polyclonal anti-TACI (Abcam) with secondary anti-rabbit Ig-DiLight649 (Jackson Immunoresearch, West Grove, PA) and the appropriate isotypes. Intracellular TACI expression was determined after treatment with permeabilization reagents.

For calcium assays, fresh peripheral blood mononuclear cells (PBMC) were incubated with Fluo-3, Fura Red (both Molecular Probes) (30 min) and CD19 (10 min) at 37°C. Cells were washed twice and resuspended in Hank's balanced salt solution (HBSS) supplemented with fetal calf serum (FCS). Cytosolic calcium levels were measured on a Facs Canto II; after 1 min, 20 µg/mL IgM F(ab')2 fragments (Jackson Immunoresearch) were added. After another 5 min, 2 ug/mL ionomycin (Calbiochem, San Diego, CA) was added. Area under curves were calculated using Facs Diva and Prism software. Next, LCL were stained and resuspended in calcium-free phosphate buffered saline (PBS). Calcium chloride as added after intracellular calcium storage depletion with ionomycin or thapsigargin (Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro stimulation assays and PCR

LCL were cultured in RPMI 1640 containing 10% FCS at the subsequent conditions: mouse anti-TACI (0.5 µg/mL, R&D systems, Minneapolis, MN) and goat anti-mouse IgG microbeads (Miltenyi Biotec, Carlsbad, CA) with or without IL-4 (100 U/mL, Im-

munotools); CpG phosphorothioate-modified oligodeoxynucleotide 2006 (0.5 µg/mL, Alexis Biochemicals), IL-10 (10 ng/mL, Becton Dickinson), or anti-CD40 (0.1 µg/mL,R&D systems). After 48 hours, cells were lysed and total mRNA was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Reverse transcription was performed using an iScript cDNA synthesis kit (Biorad, Hercules, CA). Primers were mixed with IQ SYBR green supermix (BioRad). The detection run started at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Assays were performed in duplicate or triplicate in 96-well plates using C1000 Thermal Cycler (BioRad). Results were normalized to the endogenous GAPDH mRNA ($2^{-\Delta CT}$). The following primers were used: GAPDH Forward: 5'-GTCGGAGTCAACGGATT-3'; GAPDH Reverse: 5'-AAGCTTCCCGTTCTCAG-3'; AICDA Forward: 5'-TGCTCTTCCTCCGCTACATCTC-3'; AICDA Reverse: 5'-AACCTCATACAGGGGCAAAACC-3'; NFκB Forward: 5'-GAAGCACGAATGACAGAGGC-3'; NFκB Reverse 5'-GCTTGGCGGATTAGCTCTTT-3'; NFAT Forward 5'- GCAGAGCACGGACAGCTATC-3'; NFAT Reverse 5'-GGGCTTTCTCCACGAAAATGA-3' (All Sigma-Aldrich).

RESULTS

TNFRSF13B compound heterozygous variants in asymptomatic siblings

Analysis of the patient's *TNFRSF13B* gene showed two variants C104R and A181E. Subsequently, her family was investigated, revealing that each parent carried one variant (*figure 1, A*). The brother had normal TACI alleles and was not further investigated. The two sisters (sibling 1 and 2) however were also compound heterozygous for A181E/C104R. Sequencing of the complete *TNFRSF13B* gene showed no additional mutations, polymorphisms or alternative splicing variants.[29]

Clinical and immunological evaluation of family members

Further studies were performed to determine the role of these TACI variants. Clinically, there were no signs of immune disease amongst the family members. Of note, sibling 1 and 2 did not have recurrent infections, autoimmunity or lymphoproliferative abnormalities. Serum Ig levels were normal in both parents and sibling 2, apart from a modestly decreased serum IgA (*table 1*). In contrast, sibling 1 was hypogammaglobulinemic, albeit modestly in comparison to the index patient.

To study specific antibody synthesis, parents and siblings were vaccinated with 23-valent Pneumovax®. Parents and sibling 2 had adequate T cell-independent responses *in vivo* to pneumococcal polysaccharides, while the response of sibling 1 was suboptimal (*table 1*). Immunological evaluation was extended in sibling 1, revealing an increase in $CD8^+$ T cells. Total B cells were increased (21.7% of lymphocytes, 512 cells/µL), but the composition of the B cell compartment remained normal; notably, numbers of class-switched and non-class-switched memory B cells were within normal

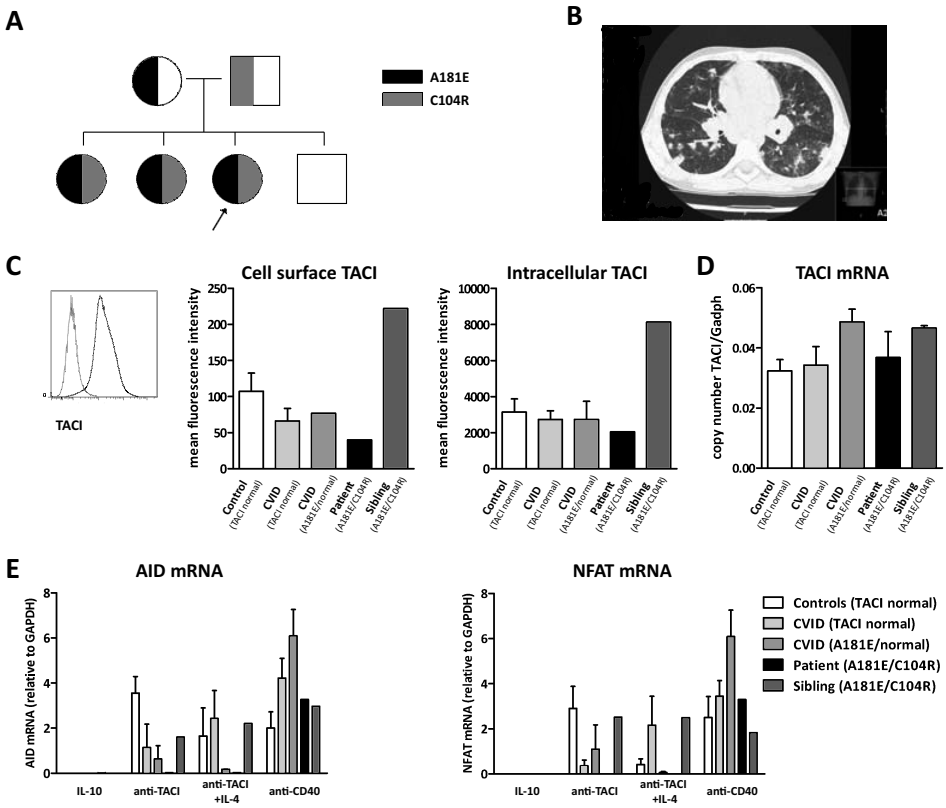

Figure 1: TACI expression and function are decreased in the CVID patient compared to her asymptomatic sibling. A, Pedigree of the studied family. Circles display females, squares display males. The index patient is indicated with an arrow. B, Chest high-resolution computed tomography scan of the index patient, revealing peribronchial consolidations with ground glass appearance and peribronchial thickening. C-D, TACI surface *(C ii)* or intracellular *(C iii)* protein and mRNA *(D)* expression. Light grey line indicated isotype, dark grey TACI *(C i)*. Results of 5 healthy donors, 3 CVID patients with normal TACI, 2 CVID patients with monoallelic A181E, the index patient and sibling 2. For D, average results of 3 independent experiments are shown. E, AID *(left)* and NFAT *(right)* mRNA induction after 48h culture of LCL with the indicated stimuli. Bars represent means ± standard error of the mean. TACI=transmembrane activator and calcium modulator and cyclophilin ligand interactor

range. Proliferative T cell responses *in vitro* were normal to mitogens, but partially impaired to recall antigens, similar to the index patient.

During the course of these investigations, sibling 1 slowly developed clinical symptoms, consisting of diarrhea, fatigue and persistent rhinosinusitis that was refractory to antibiotic prophylaxis. Splenomegaly was found on abdominal echography. Based on these findings, immunoglobulin replacement therapy was initiated, which led to significant clinical improvement. Further studies were therefore focused on the index patient and asymptomatic sibling 2.

Table 1: Immunological characteristics of patient and family.

	Patient	Sibling 1	Sibling 2	Mother	Father
Gender	♀	♀	♀	♀	♂
Age, years	11	16	14	43	43
TACI variant	C104R+A181E	C104R+A181E	C104R+A181E	A181E	C104R
Infections	yes	initially no, later yes	no	no	no
IgG	2.30 ↓	4.87 ↓	8.37	14.2	11.4
IgA	0.12 ↓	0.36 ↓	0.45 ↓	1.70	1.10
IgM	0.20 ↓	0.59 ↓	0.92	1.40	0.78
IgG1	1.50 ↓	3.30 ↓	5.7	9.80 ↑	7.60
IgG2	0.11 ↓	0.50 ↓	1.27	3.36	2.08
IgG3	1.66 ↑	0.52	0.54	1.00	0.87
IgG4	<0.07	0.04 ↓	0.18	0.05	0.24
Specific Ab titer after vaccination Pneumococcal serotype	(IU/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)
1	1 ↓	1.4	7.2	15	>40
3	2 ↓	1.2	6.1	7.0	2.6
4		0.460 ↓	6.2	0.89 ↓	1
5		4.0	0.34 ↓	2.4	6.9
6B		0.050 ↓	1.5	11	0.65 ↓
7F	2 ↓	1.7	19	1.2	15
9V		0.970 ↓	6.6	6.9	4.5
14		3.1	>40	11	39
18C		0.490 ↓	0.70 ↓	3.7	5.2
19F		0.480 ↓	>40	39	12
23F		1.8	1.4	23	11
Anti-Hib IgG	31	>100	>100	8.7	1.6

Numbers with ↑ indicate values ≥2 standard deviations (SD) above the references values; ↓ are numbers ≤ 2 SD age-related references values. ♀=female, ♂=male, Ab=antibody, Ig=immunoglobulin, TACI=transmembrane activator and calcium modulator and cyclophilin ligand interactor, Hib=*Haemophilus influenzae* type B.

TACI protein expression and function are decreased in the CVID patient but not in the asymptomatic sibling

Lymphoblastoid cell lines (LCL) were generated of the index patient and sibling 2, and compared to cell lines of 5 healthy controls, 3 CVID patients with normal TACI and 2 CVID patients with a monoallelic A181E variant. LCL of all subjects exhibited TACI expression on the cell membrane. Interestingly, the index patient's TACI expression was lower in comparison with the other subjects, especially with sibling 2, whose LCL repeatedly displayed the highest cell surface expression (*figure 1, C*). Subsequently, intracellular TACI was measured to study the possibility of impaired transition of TACI to the cell surface, and ensuing intracellular TACI accumulation. Intracellular TACI however correlated to the levels of cell surface TACI (Pearson's correlation r=0.70, p=0.037) and was also repeatedly relatively lower in the index patient (*figure 1, C*).

Basal TACI mRNA levels varied within a similar range for all LCL, including the index patient and sibling (*figure 1, D*).

Next, we investigated TACI function by measuring its ability to induce activation-induced cytidine deaminase (*AICDA*) gene transcription. The *AICDA* gene product activation-induced deaminase (AID) is pivotal for secondary antibody diversification.[36] Healthy control and CVID patient B cells with normal biallelic TACI showed AID mRNA induction upon stimulation with agonistic anti-TACI (*figure 1, E*). CVID patients with monoallelic A181E variants showed minimal *AICDA* gene transcription under those conditions. The index patient had no *AICDA* gene transcription upon TACI stimulation, while AID mRNA levels of sibling 2 B cells were comparable to B cells expressing normal TACI. The same pattern was noticed for NFAT mRNA induction; gene transcription upon TACI triggering was lower in the index patient compared to sibling 2. Stimulation of the CD40 pathway showed adequate induction of AID and NFAT mRNA in the index patient. IL-10 treatment was included as negative control that does not induce *AICDA* gene expression. Taken together, TACI protein expression and function was higher for healthy sibling 2 than for index patient B cells.

Studies of complementary B cell signaling pathways suggest compensatory TLR9 protein expression and function in the asymptomatic sibling

We hypothesized that the index patient had an additional immune defect, which in combination with the TACI variants would lead to CVID disease.

First, as defects in the B cell receptor (BCR) and its co-receptor complex have been described,[37-40] we screened the BCR signalosome. CD19, CD21 and CD20 cell surface expression of the index patient were comparable to the other LCL (data not shown). Functionally, early B cell activation seemed intact, as measured by BCR-mediated calcium mobilization in fresh PBMC (*figure 2, A*). The index patient showed a normal initial but rapidly declining calcium influx, as previously observed in CVID patients with autoimmune symptoms (own unpublished observations), and area under curves were similar to controls. As the patient's endoplasmic reticulum (ER) calcium storage capacity was not decreased compared to sibling 2 either, an additional defect in BCR signaling was considered improbable.

Second, the classical route of Ig class-switching is via CD40 with CD40L on T cells, and TACI and CD40 signaling may act in synergy.[17;19] CD40 cell surface expression (*figure 2, B*) and CD40-mediated gene transcription (*figure 1, E and figure 2, C*) were comparable between all LCL, suggesting that CD40 signaling in the index patient B cells was intact, at least in this assay.

Third, we explored TLR9 signaling, as TLR9 defects have been described in CVID[2;3] and TLR9 and TACI employ common downstream signaling pathways.[19;20] Noticeably, TLR9 expression was almost two-fold higher in sibling 2 compared to other LCL, and this increased expression was accompanied by an increased TLR9-mediated AID induction (*figure 2, C*). Thus, sibling 2 displayed increased TLR9 expression and function, possibly contributing as a compensatory mechanism for altered TACI A181E/C104R protein, in restoring the B cell overall capacity to transmit signals towards NFAT and AID.

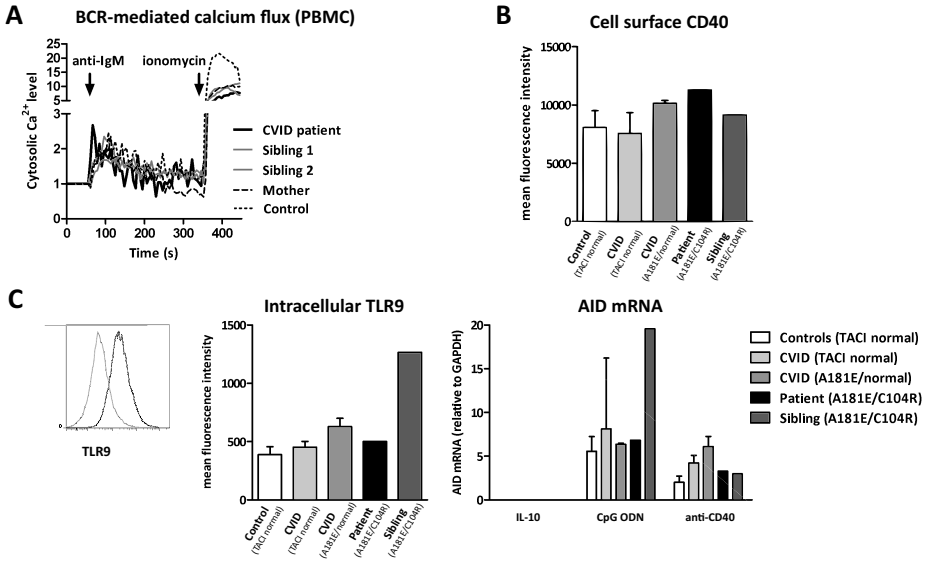

Figure 2: Similar BCR signaling but possibly enhanced TLR9 signaling in the healthy sibling. A, Calcium mobilization upon triggering of the BCR using IgM F(ab')$_2$ fragments in freshly isolated PBMCs. B, CD40 surface expression as measured by flow cytometry. C, TLR9 B cell expression *(C ii)* and function *(C iii)*. Light grey line indicated isotype, dark grey TLR9 *(C i)*. Bars represent means ± standard error of the mean. ER=endoplasmic reticulum, BCR=B cell receptor, TACI=transmembrane activator and calcium modulator and cyclophilin ligand interactor.

DISCUSSION

We here describe differential immunological phenotypes displayed by siblings with identical genetic TACI variants. This family provides the unique opportunity to study the role and significance of the human C104R and A181E variants *in vivo* and *in vitro*. This study is the first, to our knowledge, to describe the immunological features of (compound) heterozygosity for the TACI variants C104R and A181E.

The index patient had severe CVID, which gradually evolved to late onset combined immunodeficiency.[41] Sibling 1 did eventually develop a milder form of CVID. To date, sibling 2 remains free of symptoms and has excellent specific antibody responses overall. Previous studies have shown that C104R abrogates ligand binding due to haploinsufficiency, whereas in the A181E variant, ligand binding remains intact.[28;31] At least a proportion of A181E/C104R TACI trimers will thus be able to bind ligand. As NFκB activation is however strongly decreased in both variants,[31] we hypothesized TACI function to be affected. AID and NFAT mRNA induction were reduced in the index patient, and seemed also reduced in the CVID patients with a heterozygous A181E variant.[20] Conversely, sibling 2 had AID and NFAT induction comparable to healthy controls and CVID patients with unaffected TACI alleles. NFAT activation induces transcription of cytokine genes encoding for example IL-4, which is involved in class-switch recombination. AID mRNA and protein expression are induced by IL-4

and CD40 ligation, and depend on STAT6 and NFκB.[43] AID is pivotal for class-switch recombination and somatic hyper mutation of the BCR;[44;45] both mechanisms are routinely impaired in CVID.

The differential TACI function in patient and sibling was accompanied by a change in TACI protein levels, showing increased TACI expression in the sibling both at the cell surface and intracellular level. TACI protein levels in the healthy sibling were increased in comparison to healthy controls as well. As *TNFRSF13B* gene transcription was comparable between all subjects except for the healthy sibling, these observations suggest that protein turnover in the healthy sibling may be decreased, possibly due to alterations in post-translational modification of TACI protein, affecting protein stability and folding, transport or degradation. The assembly of TACI into oligomers, which is dependent on CRD1, already occurs in the ER in the absence of ligand.[28] Both index patient and sibling 2 had an intact CRD1; however, additional alterations in the oligomerization process in the ER may be present in the index patient.

Alternatively, altered TACI function in the healthy sibling was compensated by increasing the TACI protein quantity via cross-talk with related immune pathways. CVID is generally assumed to be a polygenetic disorder and it is plausible that alterations in the index patient were not limited to the *TNFRSF13B* gene. This prompted us to screen other essential immune pathways in B lymphocytes and we investigated CD40, BCR and TLR9 signaling. We did not find any additional defect in BCR or CD40 signaling. Nevertheless, for both pathways, defective interaction with other cells or factors, such as abrogated CD40L ligation on T cells or CD21-C3d interactions, cannot be excluded. In contrast, TLR9 expression and function were considerably dissimilar between index patient and sibling 2. Since the TLR9 expression and function were comparable between the index patient and the other subjects, but higher in sibling 2, we hypothesize that compensatory TLR9 expression occurs in the sibling. Similarly, increased TACI protein stability may participate in a compensatory mechanism in the healthy sibling, possibly related to increased TLR9 signaling. TLR9 recognizes unmethylated CpG-motif-containing bacterial or viral DNA[46;47] and has a critical role in the prevention of several bacterial and DNA viral infections.[46-51] Ligation of TLR9 induces recruitment of MyD88[52;53], initiating several signaling events which eventually result in activation of NFκB and AP-1.[54] TLR9 engagement induced TACI upregulation and enhanced TACI-mediated class-switching of effector B cells; furthermore, it increased IgG secretion in cells exposed to anti-TACI and IL-10, and induced the secretion of IgA. Hence, compensatory TLR9 signaling could possibly balance altered TACI function in asymptomatic individuals.

This study has some limitations. The effects of TACI malfunction on expression and function of several other targets could have been addressed, including other TNF receptors and their ligands, or signaling proteins downstream of TACI, such as TRAFs, CAML and AP-1. Additional defects may not be limited to B lymphocytes. TACI expression can be induced in $CD4^+$ and $CD8\ T^+$ cells;[4;55] study of TACI-related T cell function would be particularly interesting since the patient demonstrated chronic viral infections *in vivo* and impaired antigenic T cells responses to several viruses *in vitro*.

Taken together, we here describe the diverse clinical and immunological characteristics in siblings with an identical TACI genotype. These clinical differences are supported by differences in TACI protein expression and function. Our data suggest that compensatory regulatory mechanisms involving post-translational modification of TACI protein and TLR9 exist that may overcome the altered function of TACI trimers in asymptomatic TACI A181E/C104R individuals. Additional studies of TACI protein homeostasis and its interaction with TLR9 are therefore advisable. These findings may be beneficial for stratification of CVID patients, to allow for improved personalized treatment options.

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PART IIa

PULMONARY COMPLICATIONS IN PEDIATRIC CVID DISORDERS

7

DETECTION OF PULMONARY COMPLICATIONS IN COMMON VARIABLE IMMUNODEFICIENCY

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ABSTRACT

Pulmonary complications are frequently observed in common variable immunodeficiency (CVID). We reviewed the literature related to radiological imaging techniques and pulmonary function tests (PFT) for diagnosing pulmonary complications in CVID. Scientific publications related to CVID (or primary hypogammaglobulinemia), pulmonary complications, PFT, chest X-ray (CXR) and high-resolution computed tomography scan (HRCT) were detected in PubMed, Embase and in reference lists of selected articles. Twenty-six articles including 1047 patients (587 patients with CVID) were reviewed. Up to 73% of CVID patients develop chronic structural pulmonary complications, of which bronchiectasis and bronchial wall thickening are most frequently detected. HRCT is the most sensitive method for identification of structural abnormalities, detecting pulmonary complications that were missed on CXR and PFT in 2%-59% of patients. On PFT, obstructive flow-volume curves were most commonly found, eventually occurring in 50%-94% of patients. HRCT is an important diagnostic tool for pulmonary complications in CVID at the time of diagnosis and at regular time-points during follow-up, with the proper follow-up interval yet to be determined.

INTRODUCTION

Common variable immunodeficiency (CVID) is a primary immune deficiency characterized by B cell dysfunction, resulting in hypogammaglobulinemia.[1] Other immune deficiencies with B cell dysfunction include X-linked agammaglobulinemia (XLA), IgA deficiency, IgG subclass deficiency, functional defects in antibody production and transient hypogammaglobulinemia.[2] CVID patients suffer from recurrent bacterial respiratory tract infections and parasitic gut infections, because of antibody deficiency in serum and mucous membranes.[3-5] The main diagnostic criteria for CVID are age >4 years, decreased serum IgG, IgA and/or IgM levels (at least 2 SD below the mean for age), and decreased antibody production in response to vaccinations.[6;7] In addition, other well-defined causes of hypogammaglobulinemia must be excluded.[2;6] The prevalence of CVID is estimated to range from 1:10,000 to 1:50,000.[1;4;8] The mean age at presentation of symptoms ranges from 15 to 29 years in different studies, however CVID may be diagnosed in young children and older patients (range 4-71 years). A mean diagnostic delay of 6 years has been reported,[8] which may be as a result of the wide variety of presenting symptoms.[1] At the time of diagnosis, most patients have already suffered from recurrent bacterial pulmonary infections,[5] leading to chronic pulmonary abnormalities, such as bronchiectasis and obstructive lung disease.[3;5;9;10] Restrictive lung disease, granulomatous disease and interstitial lung disease (ILD) occur less frequently, and may also be caused by immune dysregulation associated with B cell dysfunction.[8] CVID is treated with immunoglobulin replacement therapy at regular intervals and antibiotics during acute infections. Additionally, immune suppressive therapy may be used to treat disease-related autoimmunity which is present frequently. Still, progression of pulmonary disease occurs in a considerable number of patients despite adequate treatment.[11]

As a result of improvement in therapy, survival in CVID patients has increased considerably over the past decades. A recent study by Chapel *et al.* reported a mortality rate of 25% after 30 years since diagnosis, compared to a mortality rate of 25% after only 15 years in an older cohort.[12;13] Survival in CVID patients is correlated with the degree of bronchiectasis at presentation.[13] Chest X-ray (CXR), high-resolution computed tomography (HRCT) and pulmonary function tests (PFT) are the methods currently used for the detection of pulmonary complications in CVID patients. So far, no consensus has been reached as to what the optimal strategy is for diagnosis and follow-up of chronic pulmonary disease in CVID.[14] The objective of this study is to review the current non-invasive diagnostic modalities for detection of pulmonary complications in CVID patients, and to compare findings between pediatric and adult CVID patients.

MATERIALS AND METHODS

We searched the medical scientific literature for relevant papers published in the English language up to January 2008 using the keywords 'common variable

immunodeficiency', 'primary hypogammaglobulinemia', 'lung disease', 'pulmonary complications', 'pulmonary function', 'radiology', 'chest X-ray', 'radiographic findings', and 'high-resolution computed tomography'. We used PubMed and Embase as the first step in our strategy and subsequently scanned the reference lists of the selected articles for additional references. The details of the search strategy are depicted in figure 1. Relevant articles were selected based on title and abstract. The majority of papers dealt with both CVID and related hypogammaglobulinemias; from those papers, parameters of interest were extracted separately for CVID and related diseases as much as possible. Papers without specific information on the incidence or prevalence of the parameters of interest were not included in this overview.

RESULTS

Twenty-six publications were identified dealing with pulmonary complications in CVID and primary hypogammaglobulinemias (*table 1*), describing a total of 1047 patients among which were 587 patients with CVID.

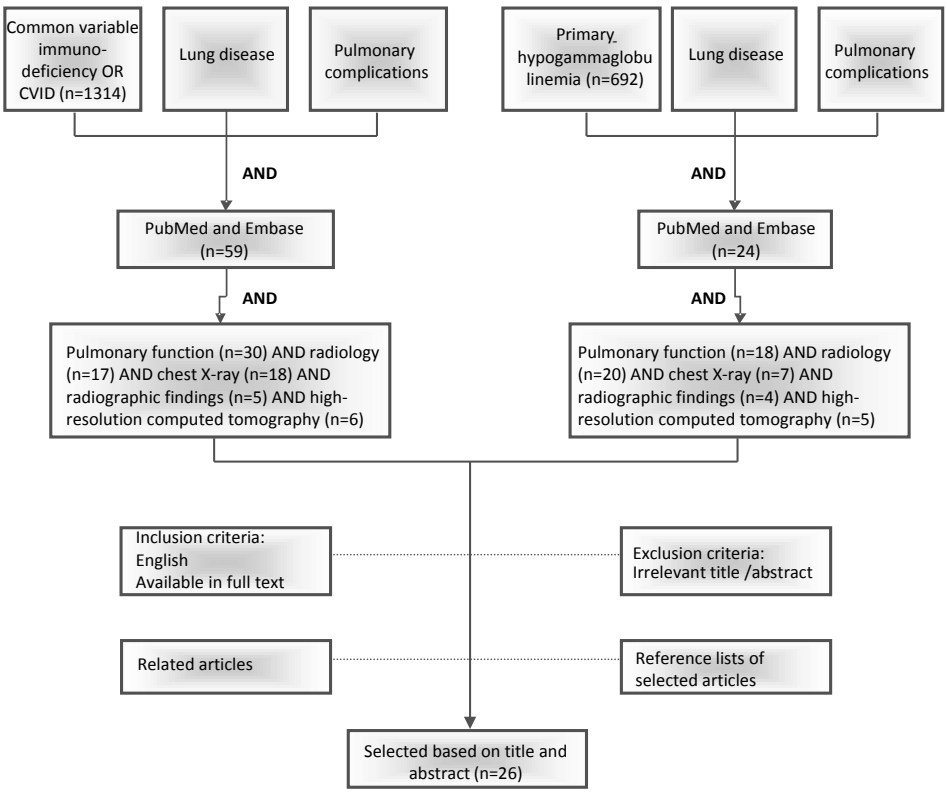


Figure 1: Flowchart of search strategy.

Table 1: Overview of reviewed articles.

Author	Patient population	Patients	Design	Objective
Adults				
Hermans *et al.* (1976)	CVID	50	Follow-up study	Evaluate the frequency of clinical associations and complications in CVID
Dukes *et al.* (1978)	CVID	50	Retrospective study	Evaluate pulmonary complications in CVID based on CXR and clinical experience
Bjorkander *et al.* (1983)	CVID, XLA	26 (24 CVID)	Retrospective study	Evaluate lung function and body growth in primary hypogammaglobulinemia
Watts *et al.* (1986)	CVID	32	Follow-up study	Evaluation of respiratory dysfunction in CVID with PFT and CXR score
Sweinberg *et al.* (1991)	CVID, XLA	22 (12 CVID)	Retrospective study	Analysis of the incidence of chronic pulmonary disease on CXR and PFT
Young *et al.* (1991)	Patients with clinical suspicion of bronchiectasis	19	Retrospective study	Evaluation of the usefulness of CT in the assessment of bronchiectasis, compared to bronchography
Curtin *et al.* (1991)	CVID, XLA	38 (28 CVID)	Retrospective cohort	Determination of the pattern of bronchiectasis on CT
Hermaszewski *et al.* (1993)	CVID, XLA, thymoma-associated hypogammaglobulinemia	295 (240 CVID)	Retrospective study	Determine clinical manifestations and complications in primary hypogammaglobulinemia
Obregon *et al.* (1994)	CVID, XLA, selective IgG deficiency, pan-hypogammaglobulinemia, chronic mucocutaneous candidiasis, selective T-cell functional defects, neutrophil defects, SCID.	46 (28 CVID)	Retrospective review	Review of CXR and CT findings in adults with primary immunodeficiencies
Feydy *et al.* (1996)	CVID, XLA, HIGM	19 (16 CVID)	Prospective cohort	Evaluation of pulmonary complications on HRCT
Kainulainen *et al.* (1999)	CVID, XLA	22 (18 CVID)	Follow-up	Evaluation of pulmonary complications, follow-up for progression
Thickett *et al.* (1999)	CVID	47	Retrospective observational cohort study	Evaluation of pulmonary complications, lung function, and HRCT findings
Martinez Garcia *et al.* (2001)	CVID	19	Follow-up study	Evaluation of pulmonary complications and response to i.v. replacement therapy
Popa *et al.* (2002)	Interstitial lung disease and Ig deficiency	29 (8 CVID)	Prospective cohort	Evaluation of the frequency and type of ILD in Ig deficiency

Table 1: Continued.

Author	Patient population	Patients	Design	Objective
De Gracia *et al.* (2004)	CVID	24	Prospective cohort	Assessment of progression of pulmonary complications in patients on replacement therapy
Park *et al.* (2005)	CVID with granulomatous lung disease or sarcoid-like CVID	18	Retrospective cohort	Evaluation of HRCT findings in CVID patients with granulomatous lung disease
Gharagozlou *et al.* (2006)	CVID, XLA	22 (13 CVID)	Observational cohort	Evaluation of pulmonary complications with HRCT and PFT
Tanaka *et al.* (2006)	CVID	46	Retrospective review	Evaluation of pulmonary complications detected on CXR and CT
Bondioni *et al.* (2007)	CVID, AG	45 (27 CVID)	Follow-up study	Assessment of pulmonary complications on HRCT
Busse *et al.* (2007)	CVID		Review	Review of pulmonary complications in CVID
Children				
Hausser *et al.* (1983)	Children with CVID	30	Observational cohort	Clinical and immunological evaluation of children with CVID
Kornreich *et al.* (1993)	Children with clinical suspicion of bronchiectasis	40	Retrospective cohort	Evaluation of the usefulness of CT in the assessment of bronchiectasis
Manson *et al.* (1997)	Children with CVID, XLA, dysgammaglobulinemia, HIGM.	37 (10 CVID)	Retrospective cohort, partially follow-up	Evaluation of the usefulness of HRCT in the detection of pulmonary complications in patients with antibody deficiencies
Newson *et al.* (1999)	Children with CVID, XLA, SCID, specific antibody deficiency, chronic granulomatous disease, hyper IgE syndrome, HIGM, Wiskott Aldrich syndrome, Schwachmann syndrome, chronic mucocutaneous candidiasis.	23 (7 CVID)	Observational cohort	Comparison of CXR and HRCT findings in children with primary immunodeficiencies
Rusconi *et al.* (2003)	Children with CVID, agammaglobulinemia (AG), IgA deficiency	24 (2 CVID)	Cohort study, partially follow-up	Evaluation of the value of HRCT in detecting progression of pulmonary complications
Abinun *et al.* (2003)	Children with CVID and XLA	29 (17 CVID)	Retrospective study	Evaluate long-term follow-up of children with primary hypogammaglobulinemia

CVID=common variable immunodeficiency, PFT=pulmonary function test, CXR=chest X-ray, HRCT=high-resolution computed tomography scan, XLA=X-linked agammaglobulinemia, HIGM=hyper IgM syndrome

Chest X-ray

Nine papers used CXR as a diagnostic method for pulmonary complications, and findings of these papers are summarized in table 2. One paper used a scoring system developed by Brasfield *et al.*, originally developed for cystic fibrosis (CF) patients.[15;16] This system scores air trapping, linear markings, pleural abnormalities, parenchymal lesions, lower lobe hyperlucency, bullous lesions, and the general severity of pulmonary complications.[16]

Findings on CXR in adults

The incidence of pulmonary complications on CXR varied predominantly with age. In adults, lung fibrosis and bronchial wall thickening were the abnormalities most frequently detected, followed by bronchiectasis and parenchymal lesions.[1;3;11;15;17-

Table 2: Overview of abnormalities found on chest X-ray.

Affected proportion (%)	Dukes *et al.*[17]	Hermans *et al.*[18]	Sweinberg *et al.*[19]	Kainulainen *et al.*[11]	Tanaka *et al.*[3]	Newson *et al.*[20]	Obregon *et al.*[21]	Thickett *et al.*[1]	Watts *et al.*[15]
Patients (number)	55	50	12	22[a]	35	25[b]	37[b]	33	30
Bronchial tree									
Bronchiectasis	38	28	50	14	29	20	32	27	23
Bronchial wall thickening					40		81		
Lobar collapse/ consolidation					17		8	3	
Air trapping						0			47
Atelectasis			17				5		
Emphysema								6	
Mucus plugs							13		
Bullae					3		3		27
Parenchymal lesions							11		30
Nodules					29				
Cavity							3	3	
Shadow								6	
Interstitial abnormalities			25						
Fibrosis				86					
GGA					17	4			
Reticulation					29				
Lymphadenopathy					14			0	
Pulmonary artery enlargement					6		11		
Hyperlucency									67
Linear markings									100

[a] Patient population with CVID or XLA. [b] Patient population included other primary immunodeficiencies in addition to CVID. GGA=ground glass appearance, CVID=common variable immunodeficiency, XLA=X-linked agammaglobulinemia, CXR=chest X-ray.

[21] Except for nodules, all abnormalities were predominantly found in the lower and middle lobes; one study reported left upper lobe predominance.[3;11] Overall, 0%-42% of CVID patients showed no abnormalities on CXR.[1;3;11;15;17] A follow-up of CXR scores was reported in only one study, which used the Brasfield scoring system. This study reported a minimal decline in CXR score after seven years.[15]

Findings on CXR in children

Two studies reported CXR findings in children. Bronchiectasis was detected in a percentage similar to that reported in adults. Contrary to findings in adults, no air trapping was reported in pediatric patients,[20] which may be due to the relatively low age of the patient population and the time at which the diagnosis was made. Abnormal findings on CXR, characteristic for chronic lung disease, were reported in all included CVID patients in a study by Abunin *et al*.[22]

High-resolution computed tomography

Over the past decades, there has been an increasing interest in HRCT for diagnosis of pulmonary complications in chronic lung diseases. Thirteen articles related to CT or HRCT were identified. Two of these studies used a CT scoring system developed by Bhalla *et al.*,[23] originally developed for CF patients.[2;24] Using this system, 25 points were assigned to patients with a normal HRCT. The severity and extent of bronchiectasis, peribronchial thickening, mucus plugs, abscesses, sacculations, bullae, consolidation, emphysema, and the number of bronchial divisions involved were scored and subtracted from 25.[23] A score <22 was considered abnormal.[2]

Bronchiectasis was most commonly detected, often in association with bronchial wall thickening and mucus plugs.[25] Both bronchiectasis and bronchial wall thickening mainly showed middle lobe predominance on HRCT, followed by the lower lobes and lingual.[3;8;20;24-26] Isolated upper lobe predominance was only reported by Obregon *et al.*,[21] and upper lobe involvement was described in two other studies in combination with diffuse bronchiectasis.[11;20] Cylindrical bronchiectasis was most commonly detected, which is the mildest form of bronchiectasis.[3;25;27;28] Figure 2 demonstrates a typical CT scan from a pediatric patient with CVID.

Parenchymal lesions such as scars and nodules were distributed randomly throughout the lungs. However, one study reports lower lobe predominance.[29] Nodules were described both in the interstitium and parenchyma, showing a bilateral distribution in approximately half of the patients.[3;11] Small nodules (<5mm) were well-defined and more randomly spread in the lungs, whereas the larger nodules usually were ill-defined and mainly found around the central bronchi.[8] Reticulation and fibrosis had middle lobe predominance.[8] Two studies found an association between ground glass appearance and parenchymal lesions, reporting ground glass appearance in all patients within this category, with middle and lower lobe predominance.[3;27] The pulmonary complications detected on HRCT are summarized in table 3.

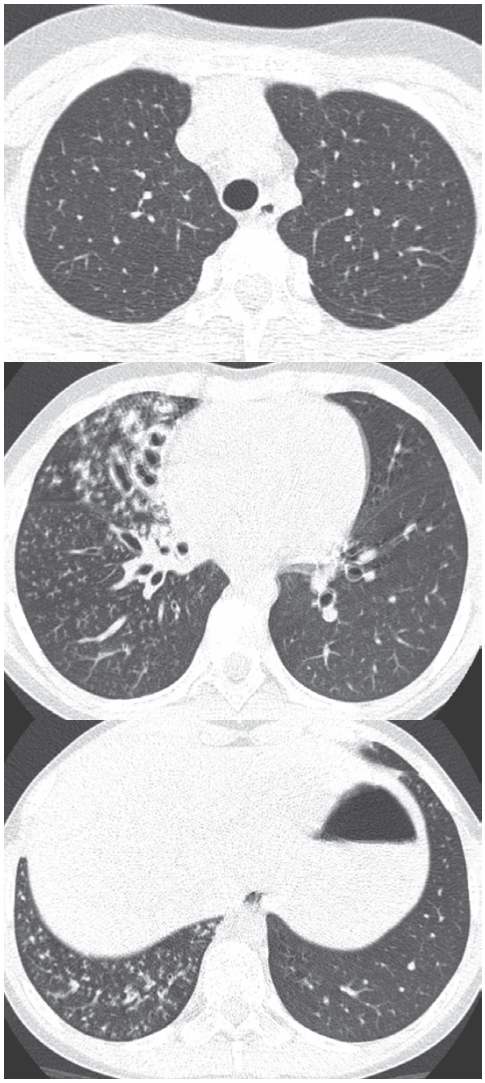


Figure 2: Twelve-year-old female with common variable immunodeficiency. Three representative CT images illustrate the relative sparing of the upper lobes, the most severe disease in the middle lobe (bronchiectasis, airway wall thickening and tree-in-bud pattern) and some involvement of the lower lobes (mild bronchiectasis on the left and airway wall thickening and tree-in-bud on the right) that is often seen in patients with CVID.

Findings on HRCT in children versus adults

Age at diagnosis or the age at the first CT scan did not contribute significantly to CT scores.[24] Progression differed between patients, although one study reports no significant progression of pulmonary abnormalities in patients with humoral immunodeficiencies over a 3-year period.[30] Incidence and severity of pulmonary complications were usually lower in children compared to adults.[24;30] Bronchiectasis and bronchial wall thickening were found in both patient groups, however, Manson *et al.* detected fewer bronchiectasis in a study with children compared with the other studies (*table 3*).[24] Severe complications such as sacculations, bullae, emphysema,

Table 3: Summary of pulmonary complications found on computed tomography scan.

Affected proportion (%)	Kainulainen *et al.*[11]	Tanaka *et al.*[3]	Curtin *et al.*[25]	Manson *et al.*[24]
Patients (number)	22[a]	30	38[a]	70[b]
Bronchial tree				
Bronchiectasis	73	40	58	29
Bronchial wall thickening			18	29[d]
Lobar collapse/consolidation		30		26
Air trapping		63 (5/8)		
Atelectasis	5			
Bullae	5	0		
Emphysema	5	0		1
Thickening of bronchovascular bundle		10		
Mucus plugs				11
Pleural thickening	36			
Interstitial lesions				
Fibrosis	81			
Honeycombing		3		
Nodules				
Effusions		20		
GGA	18	60		
Reticulation				
Lymphadenopathy		53		
Architectural distortion		13		
Upper lobe				
Middle lobe				
Lower lobe				
Parenchymal lesions				
Scars	46			
Nodules	9	83[e]		
Cysts				
Cavitations				
Pulmonary artery enlargement		17		

[a] Population consists of patients with CVID or XLA. [b] Patient population included other primary immunodeficiencies in addition to CVID. [c] In a population with granulomatous or sarcoid-like CVID. [d] Peri-bronchial wall thickening. [e] (83%=both interstitial and parenchymatous nodules, 26.7%=parenchymatous)
GGA=ground glass appearance, CVID=common variable immunodeficiency, XLA=X-linked agammaglobulinemia

Newson *et al.*[20]	Thickett *et al.*[1]	Gharagozlou *et al.*[2]	Feydy *et al.*[27]	Rusconi *et al.*[30]	Martinez Garcia *et al.*[4]	Park *et al.*[8]	Obregon *et al.*[21]	Bondioni *et al.*[29]
8[b]	34	22[a]	16	24[b]	19	32[c]	19[b]	27
63	51	59	44	71	58	38	79	38
		59[d]	38	71[d]			90	8
13	6	9	88	38				38
		9	11				37	0
	4	9		13				0
					5			
		41		21			21	
	6				16			
			32					
	2							
			13					
			6				42	
						81		
	2					38	5	
						47		
						44		
						62		
			50		26		21	33
			25			50	37	
	2							
	2						5	

and air trapping were reported rarely in children.[20;24;30] Additionally, children showed less extensive disease, not involving all lobes.[24]

Correlation between clinical symptoms and HRCT score

Various correlations between HRCT findings and clinical symptoms were reported. First of all, Rusconi *et al.* found a correlation between chronic productive cough and HRCT score, in patients with a Bhalla score ≥4 (corresponding to ≤21 according to the original Bhalla report) at commencement of the study. Patients developed pulmonary complaints mainly when productive cough was present for ≥3 times a year.[30] Feydy *et al.* reported that the annual incidence of pulmonary infections and serum levels of IgA correlated with the occurrence of airway collapse on HRCT. Furthermore, nodules and reticulation on CT correlated with the yearly incidence of infections.[8;27] Cavities or cysts on HRCT predisposed to hemoptysis.[21] Manson *et al.* found that CT scores in children correlated significantly with the duration of respiratory complaints before diagnosis, IgG levels acquired with immunoglobulin replacement therapy, and abnormal forced expiratory volume in 1 s ($FEV_{1)}$ and forced vital capacity (FVC) values on PFT.[24] Although most studies report a correlation between bronchiectasis and clinical symptoms, Obregon *et al.* did not find this association. Neither was a correlation found between bronchiectasis and PFT outcomes in this study,[21] nor between the HRCT score at diagnosis and the number of pneumonias the patient had suffered.[30]

Pulmonary function tests

We found 10 articles related to PFT. Normal PFT outcomes were detected in 27%-78% of patients.[31-33] Abnormal PFT outcomes showed either an obstructive or restrictive pattern, or a combination of both (*table 4*). Obstructive ventilation was considered to be present if FEV_1/FVC was <80% of the predicted value,[11] although several studies used a cutoff level for FEV_1/FVC <95% confidence interval in diagnosing obstructive lung disease.[15] Obstructive ventilatory defects were more often reported than restrictive defects, except for studies by Watts *et al.* and Park *et al.*[8;15] No isolated obstructive defects were found in patients with sarcoid-like CVID, and ILD in CVID has been found to be associated with restrictive abnormalities.[34] Obstructive lung complications such as asthma, chronic bronchitis and bullous lesions have been reported to develop in 50%-94% of CVID patients.[4;5;15;35] One study reports that FEV_1 was used to follow the progression of obstructive complications in patients.[19]

A restrictive ventilatory defect was considered to be present when the total lung capacity (TLC) was <1.64 x standard error of the estimated value.[15] In contrast to obstructive pulmonary complications, only 40% of the patients developed restrictive ventilatory defects.[15] Watts *et al.* mainly found restrictive lung disease in the younger patients (mean age 26 years as opposed to 37 years in the obstructive lung disease group).[15] Maximum mid expiratory flow (MMEF) was only reported in one study. This parameter may indicate early airway disease, which explains its high incidence in the study by Thickett *et al.*[1]

Table 4: Summary of abnormalities found in pulmonary function tests.

Affected proportion (%)	Bjorkander *et al.*[32]	Kainulainen *et al.*[11]	Thickett *et al.*[1]	Watts *et al.*[15]	Martinez Garcia *et al.*[4]	Sweinberg *et al.*[19]	Park *et al.*[8]	Popa *et al.*[34]	Gharagozlou *et al.*[2]	Rusconi *et al.*[30]
Patients (number)	26	13	39	25	19	12	14 [a]	24 [b]	20	21 [b]
Obstructive	31	15	18	24		58		29		19
Restrictive	8		8	40	5	17	21	100		
Obstructive & Restrictive	8			8		0	57			
Low DLCO		31			58					
Abnormal MMEF			68 (13/19)							
CAL					53					
Low FEV1			26						65	
Low FVC			13						55	
Normal FEV1/ FVC			74	36				21		

[a] In a population with granulomatous or sarcoid-like CVID and nodules and/or reticulation.
[b] Patient population included other primary immunodeficiencies in addition to CVID. DLCO=diffusion capacity; MMEF=maximum mid expiratory flow; FEV1=forced expiratory volume in 1 s; FVC=forced vital capacity; CAL=chronic airflow limitation

Correlation between PFT and clinical symptoms

Patients with restrictive lung disease were found to suffer from pneumonias more frequently while receiving immunoglobulin replacement therapy when compared to patients with obstructive outcomes, and had a longer period between the onset of symptoms and diagnosis.[19] All seven patients with a decreased MMEF, in the only paper that studied this value, suffered from chronic cough and sputum production.[1] All CVID patients with granulomatous disease and abnormal PFT outcomes suffered from shortness of breath and persistent cough.[8]

Comparison of CT, CXR and PFT

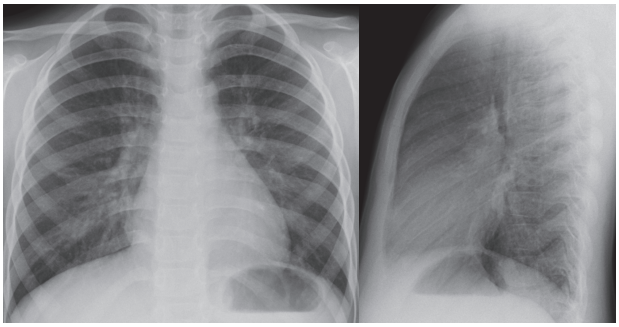
In five studies, patients underwent both CXR and HRCT, demonstrating a higher sensitivity of HRCT. Bronchiectasis was reported in all studies, ranging from 14%-32% on CXR to 32%-79% on HRCT. Bronchiectasis was found on HRCT in 27% of patients with normal CXR scores.[3] Findings of this and other pulmonary abnormalities on CXR and HRCT are compared in table 5.[1;3;11;20;21] Furthermore, HRCT and CXR were compared to PFT findings. Restrictive PFT outcomes showed an association with

Table 5: Comparison of findings on CXR and HRCT.

	Kainulainen *et al.*[11]		Tanaka *et al.*[3]		Newson *et al.*[2]		Obregon *et al.*[21]		Thickett *et al.*[1]	
Proportion affected (%)	**CXR**	**HRCT**	**CXR**	**HRCT**	**CXR**	**HRCT**	**CXR**	**HRCT**	**CXR**	**HRCT**
Patients (number)	22[a]	22[a]	35	30	25[a]	25[a]	37[a]	19[a]	33	34
Bronchial tree										
Bronchiectasis	14	73	29	40	20	32	32	79	27	71
Bronchial wall thickening			40				81	90		
Collapse/ consolidation			17	30		20	8		3	6
Emphysema		5	0	0					6	4
Air trapping				6	0	8	0	37		0
Mucus plugs							13	21		
Atelectasis		5					5			
Bullae		5	3	0			3			
Lymphadenopathy			14	53					0	3
Pleural thickening	23	36					24		9	9
Lower zone shadow									6	0
Interstitial lesions									0	3
Fibrosis	86	81								2
Honeycombing			0	3						
Effusion			23	20						
GGA		18	17	60	4					
Reticulation			29					42		
Parenchymal lesions										
Nodules		9	29	83			13	37		
Lines							16			
Scars		46						21		
Cavity							3		3	2
Cysts								53	0	2
Pulmonary artery enlargement			6	17			11			
Normal			27 (4/15)	0		28			42	24

[a] Patient population included other primary immunodeficiencies in addition to CVID; GGA=ground glass appearance, PID=primary immunodeficiency.

structural abnormalities such as fibrosis on CXR. Obstructive ventilatory defects were associated with emphysema, bullae, bronchiectasis and atelectasis.[15;19] Several studies demonstrated a moderate correlation between HCRT score and PFT. This indicates that both techniques measure different aspects of the disease and may be complementary. Structural abnormalities were more prevalent than functional defects, demonstrating the overall higher sensitivity of HRCT. MMEF, which is an early marker of peripheral airflow limitation, was found to be abnormal in some patients with normal HRCT.[1] Figure 3 demonstrates a young patient who is unable to perform the PFT manoeuvres, who has aspecific CXR findings, and who was diagnosed with bronchiectasis and atelectasis on the HRCT scan.



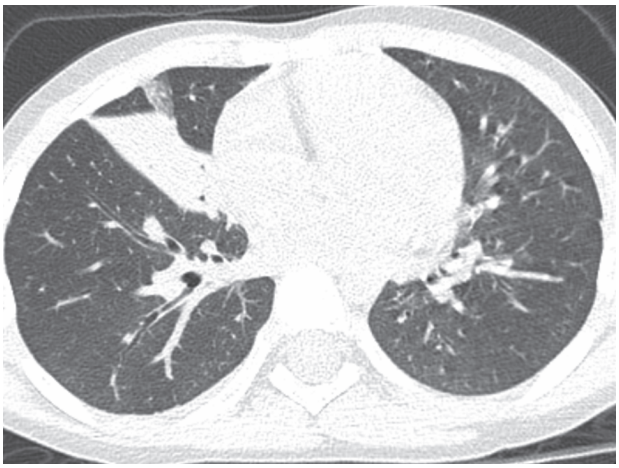


Figure 3: Female at the age of 4 years and 1 month with common variable immunodeficiency. Female patient at the age of diagnosis. At the age of 4 years, children are usually too young to perform pulmonary function test maneuvers. The chest radiograph demonstrates some aspecific increased linear markings in the right paracardiac area. High-resolution computed tomography scan demonstrates partial atelectasis of the middle lobe and cylindrical bronchiectasis in the right lower lobe.

DISCUSSION

In this review, incidence and diagnosis of pulmonary complications in patients with CVID are summarized and discussed. Pulmonary complications were found relatively often and contribute to considerable morbidity and mortality in patients with CVID. Optimal diagnosis and follow-up of these complications is likely to result in improved patient care.

Twenty-six studies were reviewed. Bronchiectasis and bronchial wall thickening were the most frequently detected complications on CXR and HRCT in both children and adults, followed by parenchymal and interstitial lesions. On PFT, obstructive lung disease was most commonly detected; restrictive lung disease and disturbed CO diffusion were also noted frequently. Sensitivity of HRCT appeared to be superior to CXR and PFT for detection of pulmonary complications in CVID patients.

About 18%- 38% of patients already had pulmonary complications at diagnosis of CVID, stressing the importance of early diagnosis.[10;36;37] Six of the reviewed studies were performed solely in children, all reporting fewer pulmonary complications in children compared to adults. This may be caused by a shorter diagnostic delay in children, a lower number of respiratory tract infections, differences in the degree of

humoral impairment, or by a lower incidence of autoimmune phenomena in children with CVID.[38] Reliable follow-up data are needed to address these issues. Of note is the fact that one study reported the presence of bronchiectasis prior to diagnosis of CVID at ages of 2 and 3 years, indicating that in rare cases, bronchiectasis develop at a very young age.[37]

Different mechanisms underlie the pathogenesis of pulmonary complications in CVID. These mechanisms include recurrent airway infections, inflammatory conditions caused by immune dysregulation (such as granulomatous disease and lymphoid interstitial pneumonia), and malignancies.[39] This is in contrast to other types of hypogammaglobulinemia, where pulmonary complications are predominantly caused by infections.[4;5;9] Infections typically cause bronchiectasis, bronchial wall thickening, mucus plugging, atelectasis and consolidations. However, there may be considerable overlap between the findings on HRCT and CXR between patients with lung pathology secondary to infections, immune dysregulation and even sometimes malignancies. For this reason, additional investigations, such as bronchoalveolar lavage or surgical sampling of affected tissue, may be indicated.[39-46] This is important as certain (rare) conditions associated with pulmonary pathology in CVID require specific therapy, e.g. lymphoma, sarcoidosis and lymphoid interstitial pneumonitis. Reported incidences of granulomatous disease in CVID range from 5%-10% in different studies.[10;41;42] The incidence of LIP was reported to be 5%.[17] Contrary to the finding that the overall incidence of malignancies in adult CVID patients varies between 11% and 16%, the reported rate of pulmonary neoplasms is likely to be significantly lower (1%), with only one study reporting on the incidence of pulmonary lymphoma.[42;44]

In view of the diagnostic possibilities for imaging of pulmonary complications, HRCT remains the most sensitive method. Drawbacks of this technique are higher costs and radiation dose, although this dose has decreased substantially over the past decades.[47] Children are more sensitive to radiation damage than adults and usually have a longer life expectancy in which radiation-related complications may develop. Additionally, increased radiosensitivity has been reported in CVID patients, when compared to healthy controls.[48] Other diagnostic methods such as CXR are safer, but fail to detect many pulmonary complications and their progression.

Considering prevention and treatment of pulmonary complications, different approaches apply, mainly directed by the underlying cause(s) of the pulmonary abnormalities. Lymphoid interstitial disease and with granulomatous disease typically responds well to immunosuppressive therapy, while pulmonary neoplasm require disease-specific treatment. Progression of pulmonary complications secondary to infections can largely be delayed by proper immunoglobulin replacement therapy and adequate use of prophylactic and therapeutic antibiotic regimens.[5;11;49;50] However, despite adequate use of these modalities, progression of pulmonary disease may still occur. Remarkably, some patients receiving immunoglobulin replacement therapy still show progression on HRCT without suffering any notable episode of pneumonia, which has been described as `silent progression`.[11;36] Silent progression was detected in CVID in various studies, and may be missed when patients are only followed via CXR, PFT or questionnaires.[11;36] Although a study by Roifman *et al.* failed to show a

significant difference in the incidence of infections between high-dose and low-dose administration of immunoglobulins,[5;50] it cannot be excluded that administration of high-dose immunoglobulin replacement could decrease the rate of silent progression. Physical exercise therapy has been proposed as supportive intervention besides immunoglobulin replacement therapy.[17;18;51] Lobectomy is a last treatment option in patients with persistent segmental pulmonary infection, particularly in areas where bronchiectasis has developed.[51]

Recent survival studies show a lower mortality compared to studies from past decades.[13] Additionally, a lower incidence of pulmonary complications is seen on CXR, PFT and HRCT in studies performed after 1995, when compared to earlier studies.[15;19;21;25;26;28] Since the median diagnostic delay has not changed much over the past decades, optimalization of treatment strategies is likely to account for this improved survival.[13;38;52] So far, no consensus has been reached as to what the optimal intervals are for follow-up of CVID-related lung complications. Longitudinal studies that include PFT and HRCT during follow-up are required for the development of complication specific guidelines.

CONCLUSION

Pulmonary complications are commonly seen in CVID and related diseases, and morbidity and mortality is relatively high. Since complications are often missed on CXR and PFT, HRCT might well be an important adjunct in the long-term follow-up in CVID patients. Complications identified on HRCT may indicate optimalization of prophylactic therapy, and the use of immunosuppressive therapy in selected patients. We suggest performing low-dose HRCT at the time of diagnosis and at regular time-points during follow-up, with the proper interval yet to be determined. PFT, although featured by decreased sensitivity for complications, can be used to monitor patients at a more regular basis.

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A CT SCORE FOR THE ASSESSMENT OF LUNG DISEASE IN CHILDREN WITH COMMON VARIABLE IMMUNODEFICIENCY DISORDERS

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ABSTRACT

Background: The prevalence and severity of structural lung disease in children with common variable immunodeficiency (CVID) disorders is not well-known, and a dedicated computed tomography (CT) scanning protocol and CT scan scoring system have not been described in this category.

Methods: Cohort study of 54 children (34 CVID, 20 CVID-like disorder) in a stable condition who underwent volumetric inspiratory and end-expiratory CT scans. Scans were scored for airway abnormalities, interstitial and parenchymal lung disease, and lymphadenopathy using a newly developed CT scan scoring system. Scores were normalized to a 0-100% scale. Observer agreement was assessed using an intraclass correlation coefficient (Ri). Prevalence and severity of CT scan abnormalities were calculated.

Results: Structural lung disease was common (85%-93%), but usually mild as reflected in the relatively low scores (bronchiectasis score 2.8% ± 6.4%). Moderate-to-severe bronchiectasis was found in 3 (5%) patients. Expiratory air trapping was the most common finding, found in 71% to 80%, but often in a mild form; application of a cut-off level of >10% reduced its prevalence to 33% to 38%. In 9% to 15% of all patients, air trapping was the only abnormality. Multiple lung nodules were seen in 24% to 25% and could disappear after corticosteroid treatment. Observer agreement was moderate (Ri 0.6-0.79) to good (Ri >0.8) for all items and the composite scores, except airway wall thickening.

Conclusion: In children with CVID disorders, mild structural lung disease is common. Expiratory CT scans show the most frequent abnormality, air trapping. The occurrence of (silent) lung disease progression and the clinical impact of CT scans require further investigations.

INTRODUCTION

Common variable immunodeficiency (CVID) is a heterogeneous immunodeficiency syndrome characterized by low levels of immunoglobulins (Ig), which makes these patients susceptible to pulmonary infections and development of chronic lung disease.[1] In addition, an increased prevalence of interstitial lung disease (granulomatous lung disease and pulmonary lymphocytic infiltration) has been described.[2-4] Prophylaxis for airway infections is given by means of immunoglobulin replacement therapy (intravenous or subcutaneous Ig), and in patients suspected of having accelerated disease progression, therapy can be intensified. Antibiotic treatment is used for both prophylaxis and treatment of established infections.[5] Physiotherapy can be of additional value.[6] Interstitial lung disease might benefit from immunosuppressive drugs, such as corticosteroids.[7-9]

The present literature suggests that lung disease is common and can be severe even in children; however, prevalence and severity of structural lung disease in children with CVID is not well known.[10-13] The prevalence of bronchiectasis in pediatric CVID varies from 29 to 71%, whereas interstitial lung disease, small airway disease (expiratory air trapping) and lymphadenopathy have solely been described in adult patients. Reports have suggested that disease can be present in asymptomatic CVID patients and that unrecognized disease progression can occur in patients known to have lung disease (silent progression).[14;15]

Because lung disease may be common and therapeutic options exist, it is important to monitor the presence and progression of pulmonary disease in CVID patients. For this purpose, it has been suggested that high-resolution computed tomography (HRCT) might play an important role,[11;16] although concerns about cumulative radiation exposure exist. Contrary tot other lung diseases, no specific CT scan scoring system for CVID exists; as a result, scoring systems originally developed for cystic fibrosis[17] are occasionally applied.[11;18;19] Scoring systems are beneficial in terms of patient follow-up and standardization in research settings. Whether the currently used scoring systems in CVID may fulfill this purpose remains uncertain, as they probably do not comprise all abnormalities reported in CVID.

At our hospital, we obtain routine low-dose chest CT scans in all children with CVID. In this report, we present the CT scan findings assessed using a standardized newly developed CT scan scoring system with the aim to supply further evidence of structural lung disease prevalence and severity in a well-defined cohort of children with CVID disorders.

METHODS

Study population

Children with clinically important humoral immunodeficiencies undergo structured follow-up at our outpatient pediatric clinic. Follow-up includes a pulmonary evaluation with chest radiograph, CT scan, and pulmonary function tests. At

present, we have followed 54 children. Thirty-four children are diagnosed with CVID according to the European Society for Immunodeficiencies/Pan-American Group for Immunodeficiency,[20] while 20 do not fulfill the complete European Society for Immunodeficiencies criteria for CVID. These 20 patients are defined as CVID-like (symptomatic selective antibody deficiency in combination with IgA deficiency and/or IgG subclass deficiency) and show similar clinical and immunological phenotypes. All CVID and CVID-like patients in this cohort are treated with immunoglobulin replacement therapy and showed significant clinical improvement after initiation of therapy. The 54 CT scans that were included in this study were obtained between June 2008 and June 2009; all were obtained in clinically stable patients (otherwise the scan was postponed). This retrospective investigation was approved by the ethical review board and informed consent was waived.

CT scanning protocol

All patients underwent chest CT scanning following a dedicated low-dose volumetric protocol on a 16-detector-row CT scanner (Brilliance-16, Philips, Cleveland, OH, USA). Scans were obtained in both inspiration and expiration by using a breath-hold instruction. Inspiratory scans were acquired in a caudocranial direction with a collimation of 16*0.75mm, pitch 0.9, rotation time 0.5 seconds, 90 kilovolt (peak) [kV(p)], and milli-Amperage per second (mA/s) depending on body weight (range 16-60 mA/s). Expiratory scans were acquired in a caudocranial direction with a collimation of 16*0.75mm, pitch 1.2, rotation time 0.4 seconds, 90 kV(p) and 11 mA/s. The expiratory scan was obtained at end-expiration.

CT image quality

Cardiac and respiratory motion artefacts were recognized in some patients, but all images could be scored. Quality of the expiration was determined by the bowing of the posterior tracheal wall in comparison with the inspiratory shape of the trachea. In two (4%) patients, the expiration was insufficient and scoring was not possible.

CT scan score

CT scans were scored for a variety of abnormalities that have been reported in CVID (*table 1*, *figure 1-8*).[16] The abnormalities (scoring system components) were assessed separately in each lobe and the lingula (the location of the lingula was assessed by using the bronchial anatomy). Severity scores for each component of the scoring system as well as composite scores (CVID-airway, CVID-parenchyma/interstitial disease and CVID-all items) were normalized to a 0-100% scale as described in more detail below. CT scan abnormalities were defined according to the Fleischner Society recommendations,[21] and some specific details are discussed below.

Bronchiectasis was defined as airway lumen diameter greater than accompanying pulmonary artery outer diameter, airways visible in the lung periphery, or as lack of normal tapering (*figure 1*). Lung periphery was defined as airway abutting the mediastinal pleura of airways within 1 cm of the costal or fissural pleura. Lack of normal

Table 1: CT scan scoring system for pediatric CVID and CVID-like lung disease.

	Score			
Measure	**0**	**1**	**2**	**3**
Inspiration (per lobe)				
Size of the largest bronchiectasis	Absent	B<2 V	B=2-3 V	B>3 V
Size of the average bronchiectasis	Absent	B<2 V	B=2-3 V	B>3 V
Extent of bronchiectasis	Absent	<33%	33%-67%	>67%
Most severe airway wall thickening	Absent	0.25-0.5 V	0.5-1 V	>1 V
Average severity airway wall thickening	Absent	0.25-0.5 V	0.5-1 V	>1 V
Extent of airway wall thickening	Absent	<33%	33%-67%	>67%
Extent of mucus plugging	Absent	<33%	33%-67%	>67%
Extent of tree-in-bud	Absent	<33%	33%-67%	>67%
Extent of opacities	Absent	<33%	33%-67%	>67%
Extent of ground glass	Absent	<33%	33%-67%	>67%
Number of lung nodules	Absent	1	2	>2
Average size of lung nodules				
Extent of septa thickening	Absent	Few, <3	Marked	Diffuse
Number of bulla/cysts	Absent	1	2	>2

	Score					
	0	**1**	**2**	**3**	**4**	**5**
Expiration (per lobe)						
Extent of air trapping	Absent	<20%	21-40%	41-60%	61-80%	>80%
Mediastinum / hilum	Short axis diameter of the largest lymph node....mm					

See also figures 1-8 for more details. B=bronchial lumen diameter, V=outer diameter of accompanying pulmonary artery.

tapering was defined subjectively; when slight lack of normal tapering (*figure 1b*) was noted in only one bronchopulmonary segment with no other signs of bronchiectasis, it was ignored. Score 1, 2 and 3 represent less than 33%, 33% to 67% and more than 67% of the lung volume involved, respectively. Within each lobe, the size of the largest bronchiectasis and the average size of the bronchiectasis were scored. Average was determined as follows: the size of each bronchiectatic airway is evaluated and the average of the severities is calculated only for the bronchiectatic airways. For example, when one out of 15 airways seen in a lobe is bronchiectatic with a severity score 3, the score for average severity is 3 and the score for most severe is also 3. Note that 14 airways are normal, which is reflected in the percentage of lung involved and not in the severity score. When three out of four airways seen in a lobe are bronchiectatic with scores of 1, 2, and 3, the score for most severe is 3 and the score for average is 2 (1 plus 2 plus 3 divided by 3).

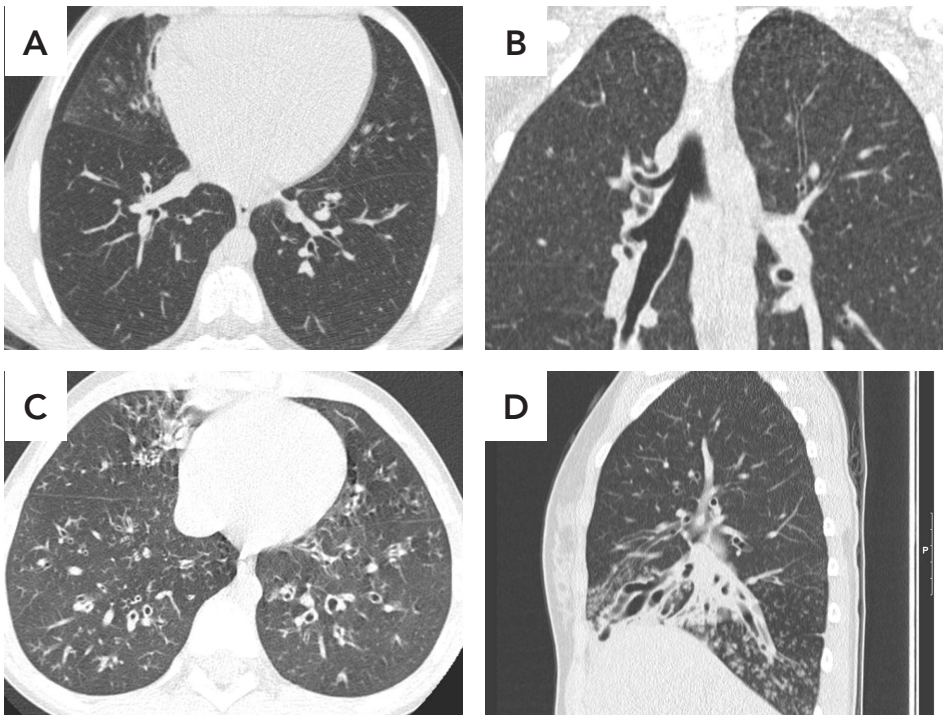

Figure 1: Bronchiectasis. **A,** This 15-year-old male patient with CVID had bronchiectasis in the middle lobe as demonstrated on this axial CT image. The airways are abutting the mediastinal pleura. **B,** This 17-year-old female patient with CVID-like disorder had a slight lack of normal tapering in the left upper lobe as demonstrated in this coronal CT image. Note the incidental finding of a tracheal bronchus on the right side. **C,** This 6-year-old male patient with CVID-like disorder had consolidation in the middle lobe, moderate and mild airway wall thickening in the lower lobes, bronchiectasis in the left lower lobe, and tree-in-bud in the right lower lobe. Note the cardiac pulsation artifact in the lingula and medial left lower lobe. **D,** This 14-year-old female patient with CVID had severe bronchiectasis (and airway wall thickening) in the middle lobe as demonstrated on a sagittal CT image.

Airway wall thickness was compared with the outer diameter of the accompanying pulmonary artery or pulmonary arteries at similar locations in the lung (*figure 2*). It has been shown that in normal infants and children, when a window level of –450 Hounsfield units and a window width of 1500 Hounsfield units were used, the ratio between airway wall thickness and arterial diameter was about 0.33.[22] We scored airway wall thickening as mild (airway wall thickness-to-artery ratio 0.33 to 0.5), moderate (airway wall thickness-to-artery ratio 0.5 to 1) and severe (airway wall thickness-to-artery ratio >1).

For mucus plugging, the percentage of airways with mucus plugs was scored (*figure 3*). For tree-in-bud, the volume percentage of the lobe that demonstrated this pattern was scored (*figure 4*). Opacities included consolidation with or without volume loss (*figure 5*). The number of lung nodules was counted per lobe (*figure 6*).

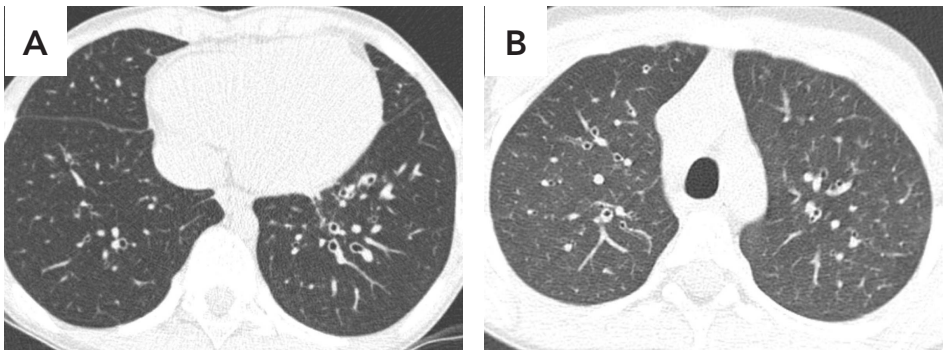

Figure 2: Airway wall thickening. **A,** In this 11-year-old male patient with CVID , mild and moderate airway wall thickening was noted in 33% to 67% of the airways in the left lower lobe. **B,** This 6-year-old male patient with CVID-like disorder (same patient as figure 1C) had mild and moderate airway wall thickening in the upper lobes.

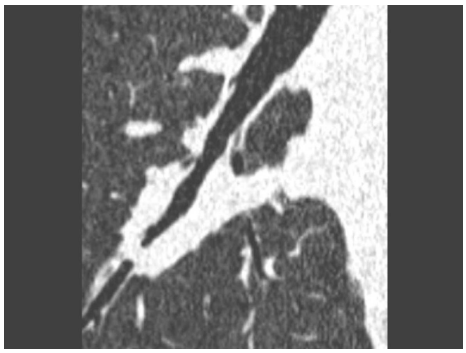

Figure 3: Mucus plugging. This 15-year-old male patient with CVID has a mucus plug in the right lower lobe as demonstrated on this multiplanar reconstructed CT image.

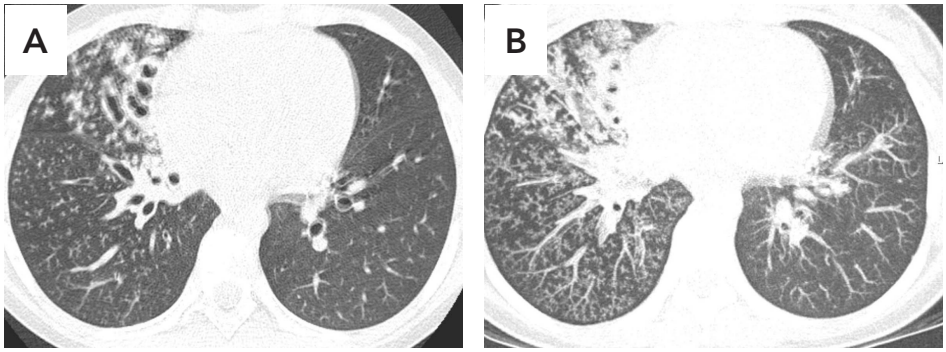

Figure 4: Tree-in-bud. This 14-year-old female patient with CVID (same patient as in figure 1D) had extensive tree-in-bud in the middle lobe and right lower lobe *(A)*. Note the value of maximum intensity projections *(B)* in the diagnosis of tree-in-bud.

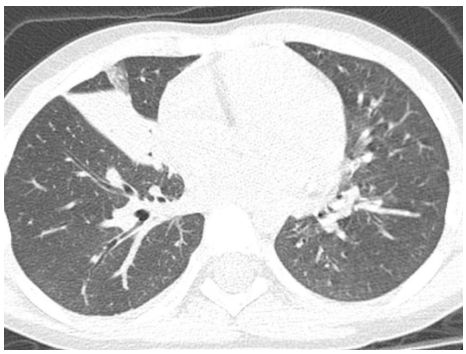

Figure 5: Opacity and ground glass. This 4-year-old female patient with CVID-like disorder had opacity (atelectasis) in the middle lobe and a small area with ground glass opacity.

The presence of only one solitary pulmonary nodule was ignored. In our experience, there is no standardized method to score the severity of septa thickening (intralobular and interlobular). For this study, we defined normal when up to three interlobular septa were visible in a lobe (*figure 7*). Scoring of mild, moderate and severe was done subjectively. Air trapping was defined as less than normal increase in attenuation of pulmonary parenchyma during expiration that followed a lobular pattern (*figure 8*). Observers ignored hyperlucent areas in the superior segments of the lower lobes and in isolated single secondary pulmonary lobules.

Calculation of the CT scan scores

CT scan scores for each scoring system component and a composite CVID airway, CVID parenchyma/interstitial and a CVID all items CT scan score were normalized to a 0-100% scale as follows. For bronchiectasis and airway wall thickening for each lobe, the most severe score (maximum 3) is added to the average score (maximum 3) and the sum is multiplied by the extent score (maximum 3). This leads to a maximum raw score of 18 points per lobe and 108 points per six lobes. To normalize the score onto a range of 0 to 100%, the bronchiectasis score and airway wall thickening score are multiplied by the normalization factor 100/108. For all other inspiratory CT scan lung items (mucus plugging, tree-in-bud, nodules, septa thickening, ground glass, opacity, bulla/cysts), the maximum score per lobe is 3 and the maximum raw score per 6 lobes is 18. To normalize these scores to a range of 0 to 100%, these scores are multiplied by the normalization factor 100/18. A combined mucus score was calculated by summing the mucus plugging scores (maximum per lobe 3) and tree-in-bud scores (maximum per lobe 3); the maximum score for the 6 lobes was therefore 36 and the normalization factor 100/36. For expiratory air trapping, the maximum score per lobe was five and the maximum score per six lobes 30. The normalization factor for air trapping was therefore 100/30. The CVID airway disease score was calculated by adding the normalized bronchiectasis score, airway wall thickening score, combined mucus score, and air trapping score. The maximum score was therefore 400 and the normalization factor 100/400. The CVID parenchyma/interstitial disease score was calculated by adding the normalized opacities, ground glass, nodules and septa scores. We excluded the

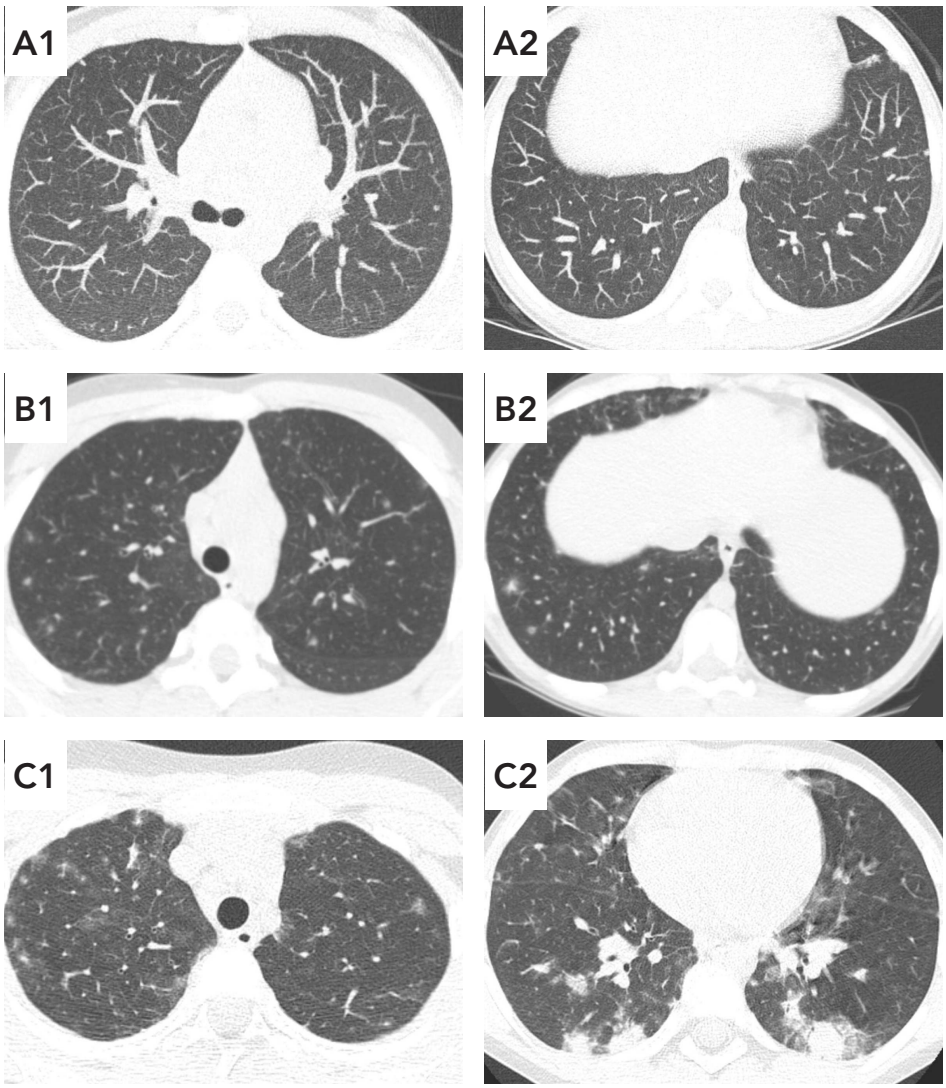

Figure 6: Multiple pulmonary nodules. **A1-2,** This 12-year-old male patient with CVID demonstrated multiple, incidentally found, well-defined small (<7 mm) pulmonary nodules as demonstrated on axial maximum intensity projection images. Most nodules were in a subpleural location. **B1-2,** This 11-year-old male patient with CVID revealed hypoechoic lesions at ultrasonography of the liver. Pathologic examination demonstrated granulomas in the biopsy tissue. Axial chest CT images demonstrate the multiple pulmonary nodules of varying size (all < 1 cm); most are ill-defined. Distribution of the nodules was random. Presumed diagnosis: multiple pulmonary granulomata or sarcoidosis-like disorder, as is known to occur in CVID. Nodules disappeared after corticosteroid treatment. **C1-2,** This 11-year-old female patient with CVID demonstrated multiple lung nodules on chest CT scan. Most nodules were ill-defined and in a centrilobular and subpleural location. The size varied; nodules in the upper lobes were smaller (approximately 5 mm) than the nodules in the lower lobes (often >1 cm). The most likely CT scan diagnosis was lymphocytic or granulomatous disease, and the nodules disappeared after corticosteroid treatment.

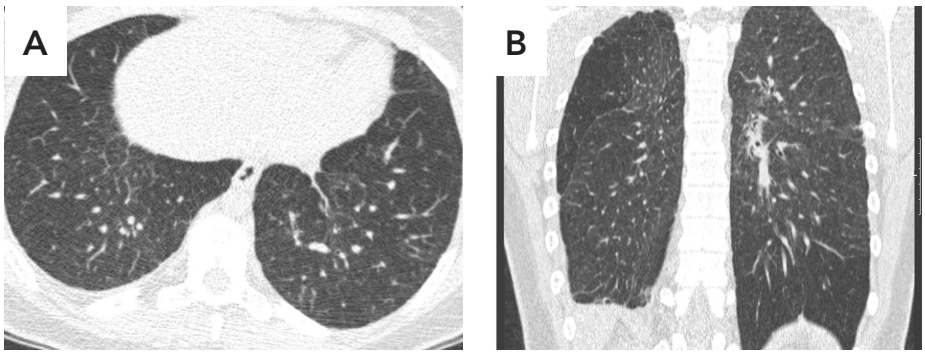

Figure 7: Architectural lung distortion and septa thickening. Axial *(A)* and coronal *(B)* CT images in a 14-year-old female patient with CVID-like disorder demonstrates distortion of the lung architecture (fibrosis) and septa thickening in the right lower lobe. Honeycombing was absent.

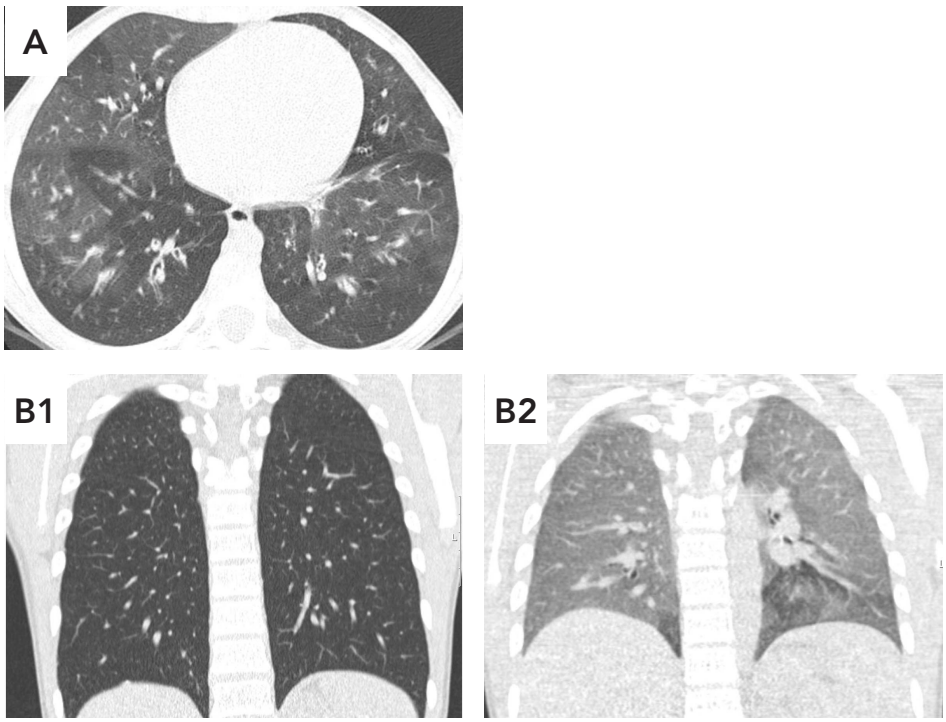

Figure 8: Air trapping. **A,** This 7-year-old male patient with CVID-like disorder had diffuse patchy air trapping. Note the characteristic sharp demarcation representing the borders of the secondary pulmonary lobules. **B1-2,** In this 13-year-old male patient with CVID, expiratory imaging demonstrated air trapping consistent with small airways disease. Inspiratory coronal CT image (*B1*); expiratory coronal CT image (*B2*).

bulla and cyst score, as this item was always scored as 0%. The theoretical maximum is 400, but in practice, ground glass and opacities/consolidations cannot be scored both for the same area in the lungs. The normalization factor that was used was therefore 100/300. Finally, an overall CVID composite score was calculated by adding the normalized bronchiectasis, airway wall thickening, combined mucus, air trapping, opacities, ground glass, septa and nodules scores. The theoretical maximum is 800, but since in practice opacities and ground glass can not be scored simultaneously in a given area we used 100/700 as the normalization factor.

The following is an example of the score calculations: A patient with CVID has bronchiectasis in the right upper lobe as the only abnormality. The largest bronchiectasis was given a score of 2, the average bronchiectasis score 2, and the extent of bronchiectasis score 3. The raw score for bronchiectasis in the right upper lobe is (2+2)*3=12, and the maximum score per lobe is 18. Because the other lobes are totally spared, the bronchiectasis score is (12+0+0+0+0+0) * 100/108 (normalization factor) = 11.1%, which is relatively low since five lobes are totally spared. The CVID airway score = 11+0+0+0 * 100/400 (normalization factor) = 2.8%, which is rather low, since none of the lobes shows evidence of bronchial wall thickening, mucus plugging or air trapping. The CVID all items score = 11+0+0+0+0+0+0+0 * 100/700 (normalization factor) = 1.6%, which is even lower given the absence of parenchymal and interstitial disease.

CT scan scoring procedure

Each scan was scored by two blinded independent observers. Scans were evaluated in the axial, coronal and sagital direction. Observers were free to change window level and width and slice thickness. All scans were evaluated in lung setting, mediastinum setting and using color coding; this last setting was used to enhance the depiction of air trapping. Maximum intensity projection imaging with a thickness of approximately 5 mm was used routinely to enhance the depiction of pulmonary nodules and to evaluate tree-in-bud pattern.

Data analysis

Observer agreement of the scoring procedure was evaluated graphically by using scatter plots with a line of identity, and intraclass correlation coefficients were calculated. An intraclass correlation between 0.6 and 0.8 represents moderate agreement, and values >0.8 represent good agreement. The prevalence of CT scan abnormalities was calculated for each observer. Mean and standard deviation of the CT scan scores were calculated as an average for both observers. CT scan scores between the CVID and CVID-like group were compared by independent samples t tests. Data were analyzed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Data are given as mean±standard deviation (SD) and (range) unless indicated otherwise.

RESULTS

Study population

The 54 children (14 girls, 40 boys) were 13±3.5 (6-18) years old. Disease duration at the time of the CT scan was 6.5±3.5 (0.1 to 13) years. Six patients were also diagnosed with asthma; no other pulmonary comorbidities were reported.

CT scan scoring observer agreement

CT scan scoring showed moderate or good reproducibility for all CT scan items and the composite CT scan scores, except airway wall thickening (*table 2*). We noted that mild bronchiectasis and mild airway wall thickening were scored more often by observer 1. Moderate and severe bronchiectasis was scored in the same three patients by both observers.

Prevalence and severity scores of CT scan abnormalities

Both the prevalence and the severity of CT scan abnormalities are summarized in table 3. Overall abnormalities were common; any abnormality was found in 85% to 93% of all patients. On the other hand, the overall extent and severity of the CT scan abnormalities was mild, the mean CVID all items score was 4.2%, reflecting that on average about 4% of the lungs showed evidence of disease. The most common abnormality was expiratory air trapping with a prevalence of 71% to 80%. In 9% to

Table 2: Observer agreement for CT scan scoring in pediatric CVID and CVID-like patients.

	Between observer agreement
Bronchiectasis	0.78
Airway wall thickening	0.51
Mucus plugging	0.79
Tree-in-bud	0.97
Lung nodules	0.82
Opacities	0.70
Ground glass	0.92
Septa thickening	0.65
Bulla and cysts	-
Lymph nodes >10mm	0.74
Air trapping	0.78
CVID airway disease score	0.83
CVID parenchyma / interstitial disease score	0.83
CVID all items composite score	0.86

Data are given as intra class correlation coefficients. No bulla or cysts were found. CVID airway disease score is a composite of bronchiectasis, airway wall thickening, mucus plugging, tree-in-bud and air trapping. CVID parenchyma/interstitial disease score is a composite of opacities, ground glass, septa thickening and lung nodules. CVID all items composite score is a composite of all items, except bulla, cysts and lymphadenopathy.

Table 3: Prevalence and severity of CT scan abnormalities in children with CVID and CVID-like disease.

	Prevalence observer 1	Prevalence observer 2	Severity score[a] (average both observers)
	Number (%)	Number (%)	Mean ± SD (maximum)
Bronchiectasis	20 (36%)	14 (25%)	2.8 ± 6.4 (37)
Moderate or severe bronchiectasis	3 (5%)	3 (5%)	-
Airway wall thickening	32 (58%)	15 (27%)	3.8 ± 5.3 (22)
Mucus plugging	7 (13%)	4 (7%)	0.8 ± 2.7 (17)
Tree-in-bud	5 (9%)	6 (11%)	2.5 ± 9.2 (50)
Multiple lung nodules	13 (24%)	14 (25%)	5.6 ± 13.8 (83)
Opacities	14 (25%)	17 (31%)	2.3 ± 3.9 (17)
Ground glass	6 (11%)	6 (11%)	1.3 ± 5.5 (39)
Septa thickening	4 (7%)	5 (9%)	0.7 ± 2.5 (17)
Bulla and cysts	0 (0%)	0 (0%)	0.0 ± 0.0 (0)
Lymph-node >10mm	5 (9%)	7 (13%)	-
Air trapping	44 (80%)	39 (71%)	10.6 ± 9.8 (33)
Air trapping >10% of lung volume	21 (38%)	18 (33%)	-
Air trapping with normal inspiration scan	8 (15%)	5 (9%)	-
CVID airway disease score (%)	47 (85%)	26 (47%)	4.8 ± 5.2 (24)
CVID parenchyma / interstitial disease score (%)	43 (78%)	28 (51%)	3.3 ± 5.6 (28)
Any disease present / CVID all items composite score (%)	51 (93%)	47 (85%)	4.2 ± 4.1 (15)

CVID airway disease score is a composite of bronchiectasis, airway wall thickening, mucus plugging, tree-in-bud and air trapping. CVID parenchyma/interstitial disease score is a composite of opacities, ground glass, septa thickening and lung nodules. CVID all items composite score is a composite of all items, except bulla and cysts and lymphadenopathy.
[a] All severity scores given are percentages. The normalization factors to obtain percentages and score calculations are described in the "Methods" section. SD=standard deviation.

15%, air trapping was the only abnormality. In these patients, 3% to 20% of the lung volume was involved. Moderate or severe disease of the large airways was uncommon (moderate-to-severe bronchiectasis was found in 5%), although bronchiectases were observed in 25% to 36%. Interstitial and parenchymal disease was fairly common; in particular, nodules were noted in 24% to 25% of the patients, while ground glass (11%) and septa thickening (7%-9%) were seen less frequently. Nodules were believed to be related to granulomatous or lymphatic infiltration. In some patients, corticosteroids were prescribed and subsequently nodules disappeared (*figure 6*). Granulomatous disease was proven in one case by liver biopsy tissue in a patient who also had multiple lung nodules. One patient presented with a solitary nodule with a reversed halo sign (*figure 9*). We found enlarged lymph nodes in 9% to 13% of our patients. There was no difference in disease severity for any of the abnormalities between CVID and CVID-like patients (p>0.23).

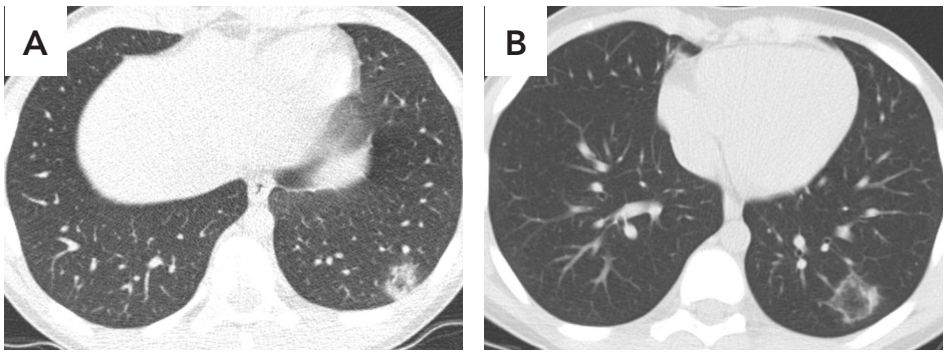

Figure 9: Incidental finding of a solitary nodule with a reversed halo sign. **A,** Axial CT scan at baseline demonstrates a solitary nodule with an inverse halo sign in an asymptomatic 14-year-old male patient. The most likely differential diagnosis was organizing pneumonia or low-grade lymphoma. **B,** Follow-up axial CT scan after 3 months demonstrates growth of the lesion in size and decrease in density, confirming the radiological diagnosis organizing pneumonia.

DISCUSSION

We systematically described the prevalence and severity of chest CT scan findings with a newly developed standardized CT scan scoring system in a well-defined cohort of stable children with CVID disorders, which were scanned using a dedicated CT scan protocol. In pediatric CVID disorders, overall disease was mild as reflected in the low severity scores, but some abnormalities were common, especially expiratory air trapping and lung nodules. Moreover, air trapping was found in some patients with a normal inspiration CT scan.

We found that in children, irreversible damage to the airways (bronchiectasis) is not very common and usually relatively mild in nature, with low severity scores, whereas severe destruction is rare. Previous studies, mostly in adults, found that CT scan abnormalities are common and often severe, although not all studies are systematic cohort studies and optimalization of treatment strategies over time may have improved lung condition.[16] The discrepancy between findings in adults and children supports active surveillance of lung disease and treatment of pulmonary infection in pediatric CVID, with the aim to prevent the development and progression of bronchiectasis. Most previous studies did not obtain expiratory CT images routinely. We found air trapping to be the most common abnormality. Air trapping is a sign of small airways disease, which might be reversible in these patients; however, this would require longitudinal studies. Also, it remains uncertain whether treatment can reverse air trapping in children with CVID. In addition, the optimal cut-off for relevant air trapping in these patients requires further study, although up to 10% air trapping was observed in nonsmoking adults.[23] We observed various patients with multiple lung nodules (*figure 6*), one patient with lung fibrosis (*figure 7*), and one patient with presumed organizing pneumonia (*figure 9*). Therefore, our data indicate that parenchymal and interstitial lung disease, which was not systematically described in children with CVID, is not a rare entity.

Our study adds important information to previous work in pediatric patients with common variable immunodeficiency disorders by Manson *et al*, Newson *et al*. and Rusconi *et al*., which describes bronchial tree abnormalities on HRCT in pediatric CVID but lacks data on interstitial and parenchymal lesions.[11-13] We studied a larger patient population with a modern CT scanner, enabling us to scan the whole lung volume instead of a sequential technique with gaps between images, as was used previously. By using a sequential technique, it has been demonstrated that bronchiectasis and other abnormalities can be missed.[24;25] We also obtained expiratory images, which were not obtained by the previous investigators. In addition, we developed a scoring system dedicated to CVID that included interstitial disease, instead of relying on CT scan scoring systems designed for cystic fibrosis. Overall, the prevalence and severity of bronchiectasis in our cohort is similar to previous studies wherein mostly mild bronchiectasis was noted in 29% to 71% of the patients.

The CT scan scoring system as described in this study can be used for future clinical and functional correlation studies. Furthermore, longitudinal studies can address the rate of (silent) disease progression, as well as reversibility of findings. Also, a CT scan score might prove useful in intervention studies in these children. Finally, impact on patient management and outcome of HRCT imaging should be addressed in future studies.

Our study has some limitations. First, the clinical role of CT imaging in these patients cannot be determined based on our study. Therefore, further studies should address this complex issue, in order to determine whether CT scanning adds relevant clinical information and whether CT scanning should be continued in the long term. The radiation dose delivered by our protocol is about 1 milli-Sievert, which is well below the annual background radiation of 2 to 3 milli-Sievert in our country. Even with this low dose, especially of the expiratory scans, the observers who scored the scans judged the levels of image noise to be acceptable for scoring the abnormalities as described in the scoring system. The described scanning protocol is our routine HRCT protocol for diagnostic purposes; also the clinical impression is that image quality is sufficient for diagnosis. Second, given the cross-sectional nature of our investigations, we cannot determine the occurrence of (silent) disease progression. Third, for some CT scan items, especially airway wall thickening, mild bronchiectasis and limited numbers of lung nodules, we found variation between the two observers. Both observers were dedicated chest imagers with 2 or 6 years of experience in academic chest radiology and hence, we feel that our variation represents variation that can be expected in a clinical setting. The described observer variation illustrates the potential for automated quantitative analysis of large[24;26;27] and small airways disease.[28]

In conclusion, we systematically described structural lung disease in a cohort of children with common variable immunodeficiency disorders. Disease was common but usually mild. Air trapping was the most common abnormality, suggesting an important role for expiratory imaging in CT scanning protocols for CVID-related lung disease. The presented scoring system covers the relevant abnormalities seen in CVID children and will now be validated in follow-up studies; subsequently, it will provide an important tool for monitoring disease progression in CVID.

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9

AIRWAY AND INTERSTITIAL LUNG DISEASE ARE DISTINCT ENTITIES IN PEDIATRIC COMMON VARIABLE IMMUNODEFICIENCY

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ABSTRACT

Common variable immunodeficiency (CVID) is a common primary immune deficiency, caused by undefined defects in lymphocyte function, and is treated routinely by immunoglobulin substitution. CVID complications include airway disease (AD) and interstitial lung disease (ILD). It was not known if AD and ILD in CVID have a common immunological etiology and should be considered separate features of the same disease, or as distinct syndromes that require specialized monitoring and treatment.

We used high-resolution computed tomography (CT) to diagnose AD or ILD in pediatric CVID patients. Spirometry and body plethysmography did not differentiate between ILD and AD. Patients with AD (n=11, 20%) developed more pneumonias while children with ILD (n=8, 15%) showed immune dysregulation characterized by autoimmune complications, more severe memory B cell reduction and expansion of non naive cytotoxic T cells. In conclusion, ILD and AD in CVID have dissimilar clinical and immunological characteristics, suggesting distinct etiology requiring tailored monitoring and treatment of these patient subgroups.

INTRODUCTION

The diagnosis of common variable immunodeficiency (CVID) is reserved for a heterogeneous patient population characterized by hypogammaglobulinemia, recurrent respiratory tract infections and several disease-related complications. [1] These complications include autoimmune phenomena and several types of lung disease, as described by Chapel *et al*.[2] The spectrum of pulmonary complications is extensive and encompasses both structural airway disease (AD), as well as interstitial or parenchymal lung disease (ILD).

Structural airway changes (e.g. bronchiectasis and bronchial wall thickening) are common in adult patients (38-79%)[3-6] and have been attributed to cumulative respiratory tract infections.[7;8] Early prevention by means of immunoglobulin (Ig) replacement therapy (IgRT) or use of prophylactic antibiotics results in a decrease in the number of lower respiratory tract infections,[9] and thereby preclusion of bronchiectasis.[10;11]

ILD includes granulomatous disease, lymphoid interstitial pneumonia (LIP), organizing pneumonia and lymphoproliferative disorders.[12] These disorders display a variety of abnormalities on computed tomography (CT) scans, such as nodules, ground glass phenomena, reticulation, bullae and cysts. ILD appears less frequently than AD[13;14] and may involve aberrant responses of the immune system,[15] suggesting a different pathogenesis. The exact mechanism(s) underlying disturbed immune responses in ILD remain unclear, although it is known that certain CVID patients are prone to develop immune-mediated complications. These include patients characterized by severely reduced class-switched memory B cells[16-18] and patients with mutations in the *TNFRSF13B* gene encoding transmembrane activator and CAML interactor, TACI.[19] The granulomatous-lymphocytic variant of ILD is associated with decreased survival[12] and its treatment includes immunosuppressive therapy such as steroids and azathioprine (reviewed in [20]), which is generally different from AD treatment. A detailed study comparing features of ILD and AD patients, however, has not yet been reported.

Most studies on pulmonary complications in CVID were performed in adult CVID patients. Reports on children are limited, and restricted to patients who already present with clinically manifest disease.[21] Those studies suggest that some pediatric CVID patients may already suffer from AD. Severity and frequency are typically lower than in adult CVID patients [22-24], possibly because children have experienced fewer cumulative respiratory tract infections than adults. ILD also occurs in the pediatric CVID population.[25]

In this study we aimed to determine whether AD and ILD are distinct clinical entities or have a common etiology by correlating radiological, functional, immunological and clinical parameters to AD and ILD in pediatric CVID patients.

METHODS

Study population

Fifty-four patients (aged 6-18 years) were included.[26] Of those, 34 patients were diagnosed with CVID according to the ESID criteria[27] and received IgRT. Twenty patients were classified with CVID-like disease, defined as selective antibody deficiency (with a less than fourfold increase of specific antibody titers upon vaccination to polysaccharide and/or to recall antigens), together with low IgA, low IgM, and/or low IgG subclass levels, or a solitary decreased IgG. These patients had recurrent infections and an inadequate response to prophylactic antibiotic treatment, which was defined by more than four breakthrough infections per year; all CVID-like patients showed marked clinical improvement after initiation of IgRT. Secondary immunodeficiencies (e.g. iatrogenic or due to enteral protein loss) were ruled out in all patients. Previous studies have shown that CVID-like patients are clinically and immunologically (e.g. B and T cell phenotype characterization) comparable to definite CVID patients.[25;26]

In June 2008, we updated our CVID monitoring protocol and intensified our pulmonary evaluation by including pulmonary function tests (PFT) and high-resolution CT (HRCT) in addition to the usual clinical evaluation and chest X-ray (CXR). All 54 patients underwent this protocol. We here report on the first evaluation round of our new protocol. The institutional review board approved this retrospective investigation and written informed consent was waived.

Clinical and immunological evaluation

Clinical data were extracted from the medical files. Pneumonia was documented when a pulmonary infiltrate was detected on conventional chest X-ray (62% of cases), or alternatively as physician diagnosed pneumonia (38% of pneumonias, following World Health Organization criteria for pneumonia and showing improvement upon treatment with antibiotics). Disease-related complications were documented according to the classification by Chapel and colleagues,[2] which includes all forms of autoimmunity, polyclonal lymphocytic infiltration, enteropathy and malignancies. Lymphocyte characteristics were compared to an immunocompetent pediatric population as described previously.[26;28] Briefly, stainings were performed on whole blood with antibodies to CD3, CD45, CD27, CD4, CD8, HLA-DR, CD38, CD45RA, CD19, CD27, CD38, CD10 (all Becton Dickinson, Krankline Lakes, NJ, USA) IgM, IgG, IgA, IgD (all Southern Biotechnology Associates, Birmingham, AL, USA) and analyzed by flow cytometry. Within the $CD19^+$ B cell compartment, the following populations were distinguished: $IgD^+CD10^+CD38^{++}$ recent bone-marrow emigrants (RBE), $IgM^+IgD^+CD27^-CD10^-$ naive B cells, non-Ig class-switched $IgM^+IgD^+CD27^+$ memory B cells, IgG^+CD27^+ and IgA^+CD27^+ memory B cells. $CD3^+$ T cells were divided into $CD4^+$ and $CD8^+$ subsets, and subsequently into $CD45RA^+CD27^+$ naive and non-naive $CD45RA^+CD27^-$, $CD45RA^-CD27^+$ or $CD45RA^-CD27^-$ T cells. TACI mutations were screened for using primers and probes specific for the p.C104R and p.A181E variant, as described previously.[26] Positive results were confirmed by sequencing of the *TNFRSF13B* gene.

Imaging

Postero-anterior and lateral chest radiographs were obtained and evaluated according to the Chrispin-Norman chest radiograph scoring system (CNS).[29;30] Low-dose HRCT scans were obtained and scored as described previously.[25] Briefly, CT scans were scored for a variety of abnormalities reported in CVID, which were assessed separately in each lobe and in the lingula. Severity scores for composite structural airway disease and composite interstitial disease were used as a reference standard to diagnose AD and ILD. The CVID airway disease score is a composite of bronchiectasis, airway wall thickening, mucus plugging, tree-in-bud, and air trapping. CVID interstitial disease score is a composite of opacities, ground glass, septa thickening, and lung nodules.

As some airway wall thickening, mucus plugging, air trapping, consolidation and septa thickening may be visible in 'healthy' individuals we defined an ILD score > 5 and an AD score > 7 as significant pathology.

Pulmonary function tests

Using Jaeger Masterscreen CS and Masterlab systems (Masterlab, Erich Jaeger, Würzburg, Germany), lung function was measured on the following parameters: percentage of predicted forced vital capacity (FVC%), percentage of predicted forced expiratory volume in 1 sec (FEV_1%), percentage of predicted maximum midexpiratory flow (MMEF%), and percentage of predicted total lung capacity (TLC%). Percentages were calculated for forced expiratory volume in 1 sec as part of the total lung capacity (FEV_1/FVC%) and residual volume as part of total lung capacity (RV/TLC%). CO diffusion capacity, corrected for alveolar volume (DLco/V_A) was measured using a standardized single breath technique (Masterlab, Erich Jaeger) and expressed as percentage predicted. To minimize bias from pulmonary co-morbidities, patients diagnosed with asthma (n=6) inhaled corticosteroids prior to spirometry. For reference values, a Dutch cohort of healthy children was used.[31]

Statistical analyses

All tests were performed two-tailed, and p-values ≤ 0.05 were considered significant. Differences in continuous data were tested using one-way analysis of variance (ANOVA) with post hoc Bonferroni tests and independent samples t tests for parametric data, or Kruskal-Wallis tests with post hoc Mann-Whitney *U* tests plus Bonferroni corrections for nonparametric data. Binary and categorical data were compared using Pearson's chi-square tests or Fischer's exact tests. Correlations were calculated with Pearson's correlation coefficients. To compare lymphocyte subsets, which is complicated by age-related changes, all values were normalized to the median value of healthy controls from the same age group.[28] The deviations of the median (100%) were expressed as percentages and used for further comparisons. All analyses were performed with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Study population

The characteristics are summarized in table 1. Apart from the anticipated difference in immunoglobulin levels, baseline characteristics were not significantly different between the CVID and CVID-like patients. A small number of children had already developed CVID-related complications, including autoimmune cytopenia and granulomatous

Table 1: Characteristics of children with CVID disorders.

	CVID (n=34)	CVID-like (n=20)	P value[a]
Male	28 (82%)	12 (60%)	0.08
Age at diagnosis, years	6.3 (2.5)	7.0 (2.7)	0.36
Age at HRCT, years	13.1 (3.3)	12.8 (3.7)	0.77
Diagnosis specification	N/A		
Specific antibody deficiency (SAD) with IgA, IgM and IgG subclass deficiency		1 (5%)	
SAD with IgG deficiency		5 (25%)	
SAD with IgA and IgG subclass deficiency		5 (25%)	
SAD with IgM and IgG subclass deficiency		1 (5%)	
SAD with IgM and IgA deficiency		1 (5%)	
SAD with IgM deficiency		1 (5%)	
SAD with IgG subclass deficiency		6 (30%)	
Ig levels at diagnosis, g/L[b]			
Serum IgG	4.5 (1.5)	7.9 (2.6)	<0.001
High	0 (0%)	4 (20%)	
Normal	0 (0%)	11 (55%)	
Low	34 (100%)	5 (25%)	<0.001
Serum IgA	0.40 (0.48)	0.57 (0.43)	0.14
High	1 (3%)	0 (0%)	
Normal	4 (12%)	14 (70%)	
Low	28 (85%)	6 (30%)	<0.001
Serum IgM	0.60 (0.51)	0.87 (0.40)	0.051
High	2 (6%)	0 (0%)	
Normal	13 (41%)	14 (70%)	
Low	17 (53%)	6 (30%)	0.011
IgG trough level during Ig replacement therapy, g/L	10.2 (2.8)	11.5 (2.8)	0.13
Chapel classification[c]			
No complications	29 (85%)	17 (85%)	0.45
One complication	2 (9%)	3 (15%)	
Two complications	3 (6%)		
TACI mutation	7 of 26 (27%)	2 of 14 (14%)	0.45
Asthma	5 (15%)	1 (5%)	0.40

[a] Independent samples t test, Pearson's chi-square or Fischer's exact test. [b] Age-related reference values for immunoglobulin (Ig) levels are provided in appendix 1. [c] Complications include autoimmunity, polyclonal lymphocytic infiltration, enteropathy and malignancies. Values are represented as mean (standard deviation, SD) or in number (percentages). As Ig levels vary with age, these are also depicted categorically: 'high' (≥+2 SD), 'normal' (−2 SD to +2 SD) and 'low' (≤−2 SD) in absolute numbers and percentages. TACI=transmembrane activator, calcium modulator and cyclophilin interactor.

liver disease. Mutations in the CVID susceptibility gene *TNFRSF13B* encoding TACI were found in both the CVID and CVID-like population.[32;33] Six patients had asthma.

HRCT scores, chest radiography findings and pulmonary function tests

HRCT abnormalities were detected in the majority of both pediatric CVID and CVID-like patients, but were usually mild.[25] There were no significant differences in HRCT scores between the CVID and the CVID-like patients.

On CXR, only mild abnormalities were detected (*table 2*). The highest Chrispin-Norman score was 6 (maximum 38), there were no large soft shadows visible, and there were no significant differences in CXR between the CVID and CVID-like population. The mean PFT results were within the normal range, and there were no significant differences between CVID and CVID-like disease. PFT and CXR scores were correlated weakly but significantly. Bronchiectasis, as detected on HRCT, had a moderate significant correlation with FVC% (r=-0.35, p=0.01), FEV_1% (r=-0.44, p=0.001), MMEF% (r=-0.38, p=0.006), FEV_1/FVC% (r=-0.29, p=0.04), as measured by PFT. Bronchiectasis was also correlated with the Chrispin-Norman score (r=0.29, p=0.04) and with mottled shadows (r=0.49, p<0.001) on CXR. Air trapping on HRCT significantly correlated with FVC% (r=-0.52, p<0.001), FEV_1% (r=-0.69, p<0.001), MMEF% (r=-0.49, p<0.001), RV/TLC% (r=0.45, p=0.001), and FEV_1/FVC% (r=-0.43, p=0.002). Airtrapping on HRCT correlated with the Chrispin-Norman score (r=0.48, p<0.001) and with mottled (r=0.55, p<0.001) and bronchial line shadows (r=0.29, p=0.04). HRCT composite scores were correlated most strongly with FEV_1% and FVC%. DL_{CO}/V_A% did not correlate with HRCT findings. Correlations between CXR and HRCT composite scores were weak to moderate (*table 3*).

Table 2: Pulmonary findings in children with CVID disorders.

HRCT airway score >7 *prevalence (%)*	20%
HRCT interstitial lung disease score >5 *prevalence (%)*	15%
Chest X ray Chrispin-Norman score	2.2 [0-6]
FVC%	94.1 [71.4-121.9]
FEV_1%	95.5 [61.9-129.1]
TLC%	94.2 [70.1-115.8]
MMEF%	99.2 [32.4-186.8]
DL_{CO}/V_A%	102.5 [66.4-158.1]
FEV_1/FVC%	89.7 [71.0-100.0]
RV/TLC%	23.4 [10.3-49.4]

Values are represented as prevalence (%) or mean [range]. HRCT=high resolution computed tomography; TLC=total lung capacity; FVC=forced vital capacity; FEV_1=forced expiratory volume in 1 second; MMEF=maximum midexpiratory flow; DLco/V_A=lung CO diffusion capacity corrected for alveolar volume; RV=residual volume.

Table 3: Correlations between high-resolution computed tomography (HRCT) composite scores and pulmonary function tests or chest radiographs.

	HRCT interstitial lung disease score		HRCT airway disease score	
	r	p[a]	r	p
FVC%	-0.44	0.001	-0.52	<.001
FEV1%	-0.47	0.001	-0.65	<.001
MMEF%	-0.24	0.08	-0.48	<.001
TLC%	-0.43	0.002	-0.10	0.49
DL_{CO}/V_A%	-0.10	0.55	0.16	0.33
RV/TLC	0.07	0.64	0.44	0.001
FEV_1/FVC%	-0.19	0.18	-0.37	0.008
Chrispin Norman score	0.33	0.02	0.45	0.001
Hyperinflation	0.21	0.14	0.11	0.47
Bronchial line shadows	0.35	0.01	0.19	0.20
Ring shadows	-0.10	0.49	-0.11	0.47
Mottled shadows	0.11	0.44	0.66	<.001

[a] Pearson's chi-square test. Significant correlations are shown in bold type. HRCT= high resolution computed tomography; TLC=total lung capacity; FVC= forced vital capacity; FEV_1= forced expiratory volume in 1 second; MMEF= maximum midexpiratory flow; $DLco/V_A$= lung CO diffusion capacity corrected for alveolar volume; RV= residual volume.

AD and ILD in relation to clinical and immunological characteristics

Both AD (n=11, 20%) and ILD (n=8, 15%) were detected in CVID as well as CVID-like patients; three patients showed a mixed pattern of AD and ILD and were included in both groups (*table 4*). Representative examples of AD and ILD are shown in figure 1 and 2, respectively.

Within the AD group, FVC%, FEV_1% and MMEF% were decreased significantly while RV/TLC% was increased. Chest radiograph scores were significantly higher than in children without lung disease. Clinically, these children had suffered more pneumonias than children without AD (91 *vs.* 45%, p=0.013) and there was a trend towards a longer disease history in AD (8.6 *vs.* 5.9 years, p=0.068). There were no differences in the distribution of gender, age, diagnosis of primary immunodeficiency, IgG trough levels or other clinical characteristics. These findings supported the notion that AD is caused by cumulative respiratory infections and we thus hypothesized that the severity of the immunodeficiency influences the extent of pulmonary complications. However, there were neither differences in immunoglobulin titers nor in absolute or relative numbers of lymphocyte subsets between patients with AD and patients without lung complications.

Within the ILD group, TLC%, FVC%, FEV_1% and MMEF% were significantly decreased, and CXR scores were higher than in patients without lung disease. Clinically, children with ILD had more CVID-related complications and thus scored significantly higher in the Chapel classification. Pediatric CVID patients with ILD showed a higher prevalence of autoimmune disease, such as autoimmune cytopenia and vitiligo, than patients without ILD (38% and 5% respectively, p=0.031). Extrapulmonary lymphoproliferative disorders

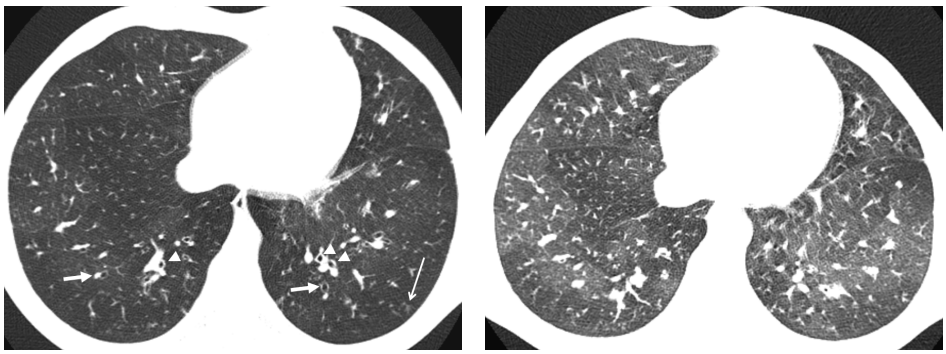

Figure 1: Airway disease on high-resolution computed tomography (HRCT). Eleven-year-old male diagnosed with CVID-like disease; six pneumonias but no CVID-related co-morbidities have been documented. **A,** Axial HRCT image in inspiration. Arrowheads represent airway with mild wall thickening. Closed arrows represent mild cylindrical bronchiectasis. Open arrow represents small area with tree in bud pattern. **B,** Axial CT image at end expiration. At both sides, areas with air trapping are visible as lucent areas.

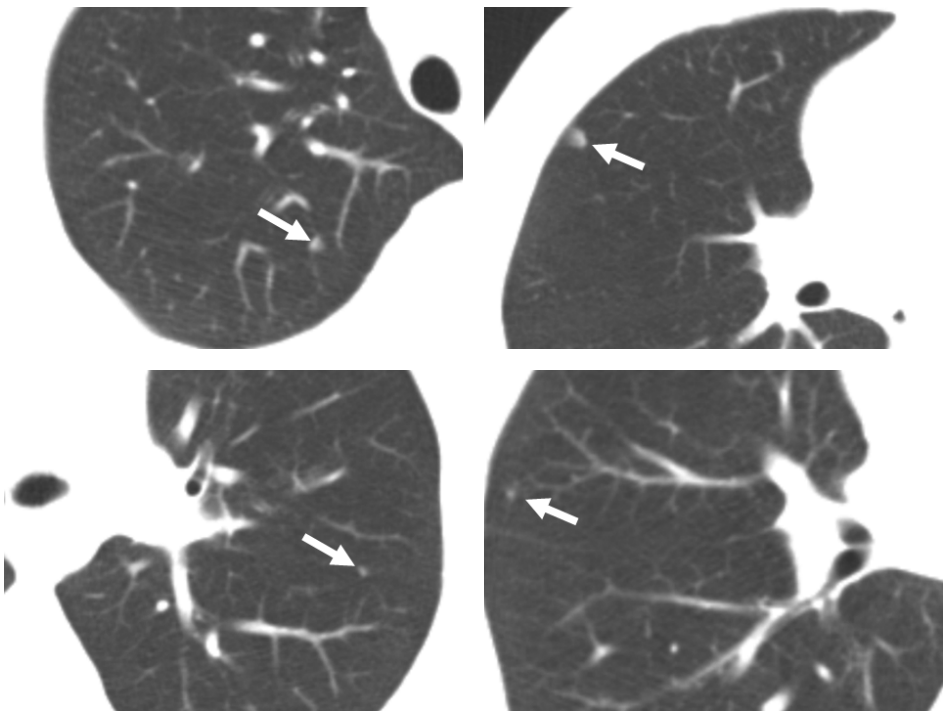

Figure 2: Interstitial lung disease on high-resolution computed tomography (HRCT). Fourteen-year-old male CVID patient with a history of systemic lupus erythematosus and vitiligo, severely reduced memory B cells but relatively high numbers of $CD8^+$ T lymphocytes. Pulmonary symptoms are limited to upper respiratory tract infections and allergic rhinitis. Four zoomed HRCT images at inspiration demonstrate the small lung nodules that were visible in this patient (*arrows*).

Table 4: Differences between patients with high-resolution computed tomography (HRCT) diagnosed interstitial lung disease, structural airway disease or no significant lung disease.

	No lung disease (n=38, 70%)
Pulmonary function test, *mean (SD)*	
FVC%	98 (9.5)
FEV1%	101 (9.5)
TLC%	96 (8.8)
MMEF%	107 (27.1)
RV/TLC%	22 (6.5)
Chest X ray, *mean (SD)*	
Chrispin-Norman score	1.7 (1.2)
Clinical data	
One or more pneumonia *n (%)*	17 (45%)
Disease duration yrs, *mean (SD)*	5.9 (3.5)
Autoimmunity[b] *n (%)*	2 (5%)
Extrapulmonary lymphoproliferative disease[c] *n (%)*	0 (0%)
Laboratory data, percent predicted[d], *median (25; 75 percentile)*	
Non Ig-class-switched memory B cells	57 (33; 88)
Ig-class-switched memory B cells	48 (36; 70)
IgG^+ memory B cells	43 (29; 68)
IgA^+ memory B cells	46 (29; 79)
$CD8^+$ T cells	117 (100; 143)
Naive $CD8^+$ T cells	91 (78; 113)
Non naive $CD8^+$ T cells	127 (76; 187)
TACI mutation	7 of 29 (24%)

[a] Three patients showed a mixed picture of ILD and airway disease and are therefore included in both columns. [b] Included systemic lupus erythematosus, vitiligo, autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, autoimmune nephritis, celiac disease, autoimmune thyroiditis and autoimmune hepatitis. [c] Included lymphadenopathy, hepatomegaly, splenomegaly, granulomatous liver disease. [d] Values are normalized to the median values in healthy children, who were divided into subgroups based on age. Values are deviations of the median and expressed as percentages. ILD=interstitial lung disease; AD=airway disease; SD=standard deviation; TLC=total lung capacity; FVC=forced vital capacity; FEV_1=forced expiratory volume in 1 second; MMEF=maximum midexpiratory flow; RV=residual volume; ND=not done; TACI= transmembrane activator, calcium modulator and cyclophilin interactor.

such as splenomegaly were exclusively found in the ILD group. Contrary to AD, ILD was neither related to the duration of hypogammaglobulinemia, nor to the occurrence of pneumonias. As interstitial changes may be caused by lymphocytic infiltrates, we explored whether lymphocytic alterations were found in the peripheral blood. Although we did not find a relation between ILD and immunoglobulin titers, there were several alterations in lymphocyte subpopulations. Percentages of B cell subpopulations revealed a significant additional decrease in class-switched (20% *versus* 48% of normal age-

Interstitial lung disease[a] (n=8, 15%)	Structural airway disease[a] (n=11, 20%)	p	Post hoc analysis p	
			ILD *versus* no lung disease	AD *versus* no lung disease
84 (7.5)	84 (9.0)	<.001	<.001	<.001
83 (10.6)	82 (13.2)	<.001	<.001	<.001
86 (6.7)	93 (10.3)	0.015	0.014	0.85
84 (34.9)	78 (33.2)	0.011	0.149	0.024
23 (6.3)	29 (9.2)	0.025	1	0.021
3.6 (1.6)	3.6 (1.8)	0.008	0.003	0.004
4 (50%)	10 (91%)	0.022	1	0.013
6.7 (3.8)	8.6 (1.8)	0.073	1	0.068
3 (38%)	2 (18%)	0.025	0.031	0.21
3 (38%)	0 (0%)	0.002	0.004	ND
19 (17; 33)	53 (10; 77)	0.024	0.008	0.77
20 (8; 28)	28 (12; 49)	0.002	0.002	0.088
9 (4; 21)	21 (12; 53)	0.002	0.001	0.166
19 (8; 42)	24 (9; 57)	0.115	ND	ND
141 (130; 221)	133 (107; 160)	0.084	ND	ND
49 (42; 86)	96 (62; 111)	0.043	0.030	0.99
262 (166; 274)	110 (69; 249)	0.041	0.030	0.99
1 of 4 (25%)	1 of 7 (14%)	ND	ND	ND

matched references values, p=0.002) and non-class-switched memory B cells (19% *vs.* 57%, p=0.008). Also, we noticed a trend in percentages of $CD8^+$ T cells. $CD8^+$ cytotoxic T cells were increased in patients with ILD compared to patients without lung disease (141% *vs.* 117% of normal age-matched references values, p=0.084). This increase was caused by a proportional increase in non naive $CD8^+$ T cells (262% *vs.* 127%, p=0.030) and appeared to be specific for $CD8^+$ T cells, as $CD4^+$ T cell subsets did not differ between the groups. Trends were similar in the absolute counts of T and B lymphocytes. The prevalence of mutations in TACI did not differ between patient groups, although sample size precluded definite conclusions. It is noteworthy that the three patients with a mixed pattern of AD and ILD did not have any CVID-related complications, although they did have the typical 'inflammatory' CVID phenotype of high RBE and low memory B cells. All had experienced one or more pneumonias and received IgRT for over 6.5 years. Furthermore, we noticed that these patients displayed T cell abnormalities, as was deducted from poor *in vivo* antigenic responses, high numbers of mainly $CD8^+$ T cells and clinically severe herpes virus infections.

Taken together, characteristics of patients with AD and ILD are remarkably different from patients without significant lung pathology, and patients with AD show different alterations than patients with ILD.

DISCUSSION

We observed that in pediatric CVID, structural airway disease and interstitial lung disease display dissimilar clinical and immunological characteristics, which may guide diagnosis and follow-up of lung pathology in CVID in the future. The pathogenesis of AD and ILD appears to be remarkably distinct. Airway disease is mainly the cumulative effect of recurrent infections and subsequent cicatrization of lung tissue, while ILD usually results from immune dysregulation, although a more local form of 'postinfectious ILD' has been suggested.[34] Indeed, in our study, patients with AD tended to have longer disease history and had a higher incidence of lower respiratory tract infections than patients without AD. Conversely, children with ILD displayed more alterations in their immune system. Clinically, this was reflected by a high occurrence of CVID-related co-morbidities, including autoimmune and lymphoproliferative disorders. These findings support previous studies reporting significant associations between (granulomatous-lymphocytic) ILD and splenomegaly,[12;35] lymphadenopathy[15] and autoimmune manifestations.[35] Immunological alterations were also noticed in laboratory results. Patients with ILD had lower percentages of memory B cells than CVID patients without ILD. Low percentages of memory B cells have been described to be a risk factor for clinical complications.[16-18;36] Additionally, we found increased numbers of non naive $CD8^+$ T cells, which include $CD45RO^+CD27^+$ memory, $CD45RO^+CD27^-$ memory effector and $CD45RO^-CD27^-$ effector T cells. The decrease in naive cytotoxic T cells is presumed to be secondary to an increased T cell proliferation and turnover. This was not reported in other papers on ILD in CVID,[34;37;38] although Mullighan *et al*.[39] found a lymphocytosis of $CD8^+CD57^+$ T cells, which are late-stage cytotoxic effector T cells. In contrast, two other studies showed a reduced number of circulating total $CD3^+$ and $CD8^+$ T cells.[12;35] These dissimilarities may be due to the study of differently defined lymphocyte (total $CD8^+$ T cells) and patient populations, namely adults with GLILD[12] or adults with pulmonary nodules.[35] Our findings may be compatible with a role for cytotoxic $CD8^+$ T cells in the pathogenesis of ILD in hypogammaglobulinemia. These T cells could be virus-specific; e.g. a role for HHV8 in the development of ILD in CVID has been suggested.[40] Further immunological studies are necessary to determine the significance of this finding.

Early and accurate detection of pulmonary complications in hypogammaglobulinemia by means of HRCT is of major clinical relevance, as both AD and ILD are treatable but require different therapies. Although PFT can also detect pulmonary disease and correlates to several HRCT findings, it has a lower sensitivity and can not accurately discriminate between AD and ILD. Arguably, the literature suggests that prophylactic antibiotics and high-dose immunoglobulin substitution therapy may prevent further progression of AD, and robust randomized-controlled trials are needed to properly address these questions (reviewed in [10]). In contrast, ILD typically responds well to immunosuppressive

therapy in symptomatic patients, and a prospective study to investigate the benefit of corticosteroids in asymptomatic CVID patients with ILD is currently being planned. In our cohort, nodules on HRCT disappeared after steroid treatment, suggesting that ILD is reversible at an early stage. Additionally, ILD appears to be asymptomatic at the initial stage, implying that screening of all (asymptomatic) CVID patients at risk would facilitate early detection and henceforth prevent progression of this disease. We propose that the immunological phenotypes of a subgroup of CVID patients may trigger more intense monitoring, and prospective structured evaluation of patients at risk for ILD can evaluate the possible yield from such a tailored follow-up protocol.

Combined AD and ILD did occur in three patients. Apart from CVID-related complications, which were not yet noted but may still develop, these patients displayed characteristics of both disease groups. It is probable that the prevalence of combined lung disease is higher in the adult population, and may rise in our cohort over time. Distinction of AD- *versus* ILD-phenotype may then become more challenging.

This study has several limitations. First, the data were collected in retrospect. Secondly, the number of patients is relatively small, and therefore our findings require confirmation. Thirdly, this study is cross-sectional and a prediction model to define which patients are at risk to develop specific pulmonary complications can be developed only in a larger cohort with follow-up.

In conclusion, we show that ILD and AD are relevant complications in children with CVID, which result from a different pathological etiology and require distinct treatment strategies. The combination clinical and immunological parameters, combined with low-dose HRCT combined with standardized scoring methods, will eventually facilitate efficient screening and follow-up for these different types of pulmonary complications in CVID. To this end, prospective follow up studies are needed.

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Appendix 1: Pediatric immunoglobulin reference values

	Reference values (g/L)		
Age (years)	**IgG**	**IgA**	**IgM**
0.5-1	2.6-15.2	0.16-0.98	0.17-1.2
1-2	2.6-13.9	0.19-1.1	0.10-0.87
2-3	4.3-13.0	0.19-2.3	0.21-0.87
3-6	5.2-13.4	0.55-2.2	0.24-1.8
6-9	5.2-14.3	0.54-2.5	0.28-1.9
9-12	5.2-15.6	0.62-3.0	0.13-1.6
12-16	5.2-15.6	0.70-3.6	0.28-2.4

Ig=immunoglobulin

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PART IIb

PATHOGENESIS OF ENTEROPATHY IN CVID AND OTHER ANTIBODY DEFICIENCIES

70

PLECONARIL-RESISTANT CHRONIC PARECHOVIRUS-ASSOCIATED ENTEROPATHY IN AGAMMAGLOBULINEMIA

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ABSTRACT

A 14-year-old common variable immunodeficiency patient developed severe protein-losing enteropathy. A chronic enteral infection with human parechovirus type 1 and norovirus was diagnosed. Treatment strategies aiming at virus eradication and providing supportive care were ineffective. The antipicornavirus agent pleconaril did not have any effect on viral replication. Symptoms improved on immunosuppressive therapy, suggesting infection-related immune dysregulation in an immunocompromised host.

INTRODUCTION

Common variable immunodeficiency (CVID) is characterized by hypogammaglobulinemia and recurrent respiratory and gastrointestinal infections.[1] These infections often have a protracted and complicated course, particularly when due to viruses that are antibody-dependent for clearance, such as enteroviruses.[2;3] Enteroviral disease in CVID can cause high morbidity and mortality.[4] Human parechoviruses (HPeVs) are a recently recognized widely circulating genus within the family of *Picornaviridae* and are closely related to the enteroviruses.[5] HPeV can cause gastrointestinal and respiratory disease in healthy individuals. Severe symptoms have been reported in rare cases.[6] Information on HPeV infections in CVID patients is scarce, which may be due to the lack of sensitivity of diagnostic tests. Molecular methods for detection of HPeVs, such as reverse transcriptase PCR, have only recently been developed[7] and are routinely used in only a few centres.

Pleconaril inhibits viral replication by binding to a hydrophobic pocket in the viral capsid of *Picornaviridae*, thereby preventing viral uncoating and viral attachment.[8] The clinical implementation of pleconaril was hampered by inconclusive results of clinical trials.[9-11] Nonetheless, pleconaril was effective in patients with potential life-threatening enteroviral infections,[12] as well as in other immunocompromised patients.[13] Its effect on the closely related HPeV remains unknown.

We report here on the effect of pleconaril administration to a CVID patient with progressive protein-losing enteropathy (PLE), in whom infection with HPeV was confirmed.

CASE

A 14-year-old boy diagnosed with CVID presented in October 2007 at our outpatient clinic with chronic diarrhea, nausea and abdominal pain. The diagnosis CVID had been made at the age of five years, based on agammaglobulinemia with normal B cell numbers, deficient antibody production after antigen and polysaccharide vaccinations, and the exclusion of other immunodeficiency. Numbers of natural killer cells, $CD4^+$ and $CD8^+$ T cells were normal and T cell proliferation upon *in vitro* mitogen or antigen stimulation was undisturbed. The patient subsequently received intravenous immunoglobulin (IVIG) replacement therapy. Immunoglobulin (Ig) G trough levels were maintained at a minimal level of 7 g/L and the patient experienced relatively few infections.

Following the initial presentation, disease progression resulted in severe PLE, causing a weight loss of 19% of the patient's body weight. Physical examination showed a cachectic boy with splenomegaly (as noted earlier). Laboratory investigation revealed mildly increased aminotransaminases (serum glutamic oxaloactetic transaminase 62 U/L and serum glutamic pyruvic transaminase 45 U/L) and decreased albumin (23.3 g/L). Haematology showed anemia (hemoglobin 7.0 mmol/L), lymphopenia ($0.71*10^9$ /L) and thrombocytopenia (platelet $102*10^9$ /L). Antibodies were found against granulocytes (anti-FcR3) and thrombocytes.

Biopsies taken from the oesophagus, antrum, duodenum, distal ileum, colon and sigmoid all showed lymphocytic infiltration. Immunohistochemistry on these infiltrates revealed a marked increase of $CD3^+CD8^-$ T cells and a lack of IgA^+CD138^+ plasma cells. Furthermore, there was duodenal villous atrophy and apoptotic colitis. Repetitive stool samples and gut biopsies remained negative for bacterial and parasitic pathogens. However, RT-PCR in stool samples and biopsies showed both HPeV and norovirus in high viral loads and 'severe PLE secondary to HPeV- and norovirus infection in an immunodeficient patient' was diagnosed.

Parenteral nutrition was started to ensure adequate administration of essential nutrients. Furthermore, attempts were made to eradicate the two viruses. As the use of pleconaril was not approved by the licensing authorities for treatment of HPeV, and our patient could not participate in any compassionate use program, alternative therapies were tried. Dose and frequency of IVIG infusions were increased, aiming to prevent viremia (IgG trough levels >20 g/L). For optimal local delivery, additional immunoglobulins were administered via a duodenal tube. Both strategies had no effect on the severity of the PLE. As Ig preparations contain predominantly IgG, and because antibody-related immunity in the mucous membranes depends mainly on IgA, other sources of IgA were explored. Human breast milk is a source of IgA and lactoferrin, the latter also a natural antimicrobial agent.[14] Breast milk of a healthy nursing woman was obtained via a voluntary donation program and tested for pathogenic microorganisms. One litre was administered daily via the duodenal tube and additional lactoferrin was given. Next, a 10-day course of the antiviral agent ribavirin was administered. Despite the abovementioned strategies, the viral loads of HPeV and norovirus in the stools remained unaltered and clinical symptoms persisted (*figure 1 and 2*).

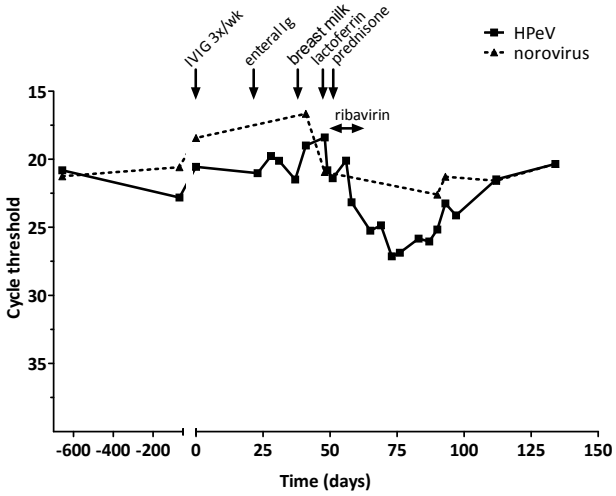

Figure 1: Fecal excretion of HPeV and norovirus. Cycle threshold (C_T) values of fecal human parechovirus (HPeV) and norovirus excretion in the case study patient during and prior to the protein-losing enteropathy. Arrows indicate the start of a new treatment strategy. Ig=immunoglobulin, IVIG=intravenous immunoglobulin substitution therapy.

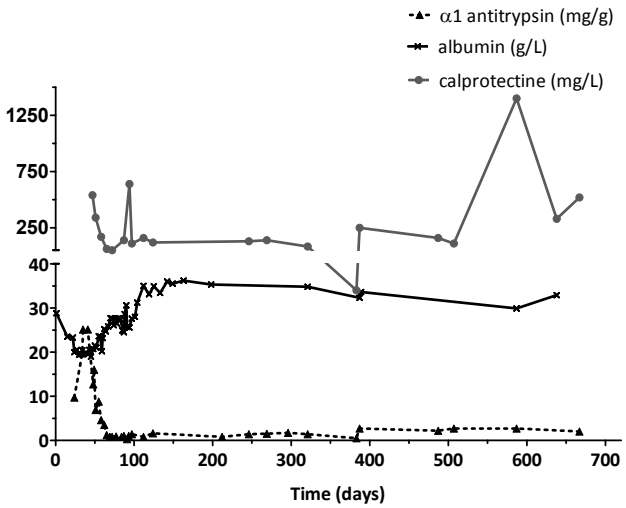

Figure 2: Laboratory parameters related to treatment. During the disease course, serum albumin (normal range 35-50 g/L), fecal α1-antitrypsin (normal range 0.1-1 mg/g) and fecal calprotectin (normal range 0-30 mg/L) were monitored to determine the extent of protein loss and intestinal inflammation. Protein-losing enteropathy is reflected in a decreased serum albumin and an increased α1-antitrypsin and calprotectin.

To confirm our working diagnosis, earlier obtained stool samples of the patient were re-evaluated. Identical strains of the currently active viruses were already present 2 years prior to current presentation; there were comparable levels of viral replication (*figure 1*) but no clinical symptoms.

The diagnosis was thus altered into 'enteropathy secondary to a perpetuated immune response and triggered by chronic viral replication in an immunodeficient host'. We initiated immunosuppressive therapy (prednisone 1.5 mg/kg), which resulted in significant amelioration of the gastrointestinal symptoms and improvement of laboratory parameters. Prednisone was tapered and tacrolimus was started; however, complete remission was not reached and viral loads remained persistently high.

In order to reach further improvement, and to prevent systemic dissemination of HPeV secondary to immunosuppressants, we decided to administer pleconaril. Reassured by appropriate chemical and clinical data in the Investigators Brochure (ViroPharm Inc. 1999; Exton, PA, United States), the drug was synthesized on request by Sequoia Research Products Ltd, Reading, UK. With the knowledge of the route of synthesis and the possible side products, the raw material was analyzed with NMR-HPLC techniques, melting point and infrared spectroscopy; a batch of 200 mg capsules was manufactured. The capsules were analyzed with ultraviolet light. The capsules fulfilled the requirements of the 'uniformity of dosage units' monograph 2.9.40 of European Pharmacopoeia.

The bio-availability was tested in a volunteer who took an oral dose of 400 mg. Plasma samples were analyzed by a liquid chromatography tandem mass spectrometry method, developed at the pharmacy of the UMCU. The method was linear between

0.1 mg/L and 15 mg/L with a limit of detection of 0.05 mg/mL. The maximum plasma concentration was 0.51 mg/L.

The patient received 400 mg pleconaril orally three times a day for 7 days. Therapeutic levels (as described for enteroviruses) of pleconaril were reached (*figure 3*). Although pleconaril was well tolerated, it did not result in a decrease of the HPeV viral load or in any further improvement of symptoms.

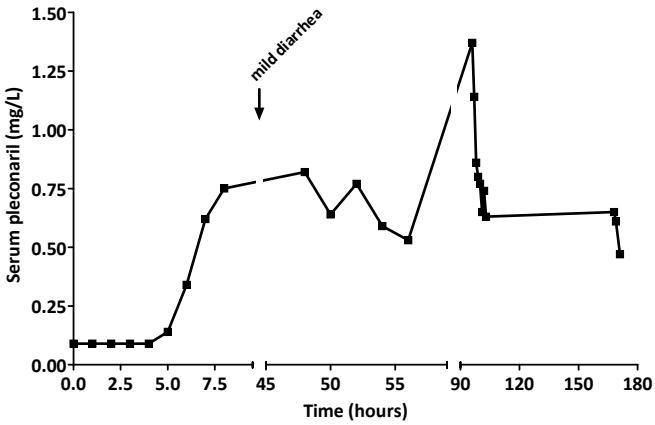

Figure 3: Serum concentrations of pleconaril. Therapeutic serum concentrations of pleconaril were reached during 7 days of administration in the common variable immunodeficiency patient. The only possible side effect was mild diarrhea on day 2.

DISCUSSION

Patients with severely decreased humoral immunity suffer from recurrent gut infections. The association between specific viral infections and severe gastrointestinal pathology in these patients is unclear, although it is recognized that immunity towards several viruses depends heavily on normal antibody production, apart from intact T cell and natural killer cell functions. Previous reports have described individual cases of chronic viral carriership in patients with humoral immunodeficiency.[15] Large surveys have not been performed, as viral culture lacks sensitivity and PCR has often not been implemented in routine practice.

However, the available data suggests that viruses might have an important role in gut pathology in specific immunodeficient patients. There are several hypotheses for the mechanisms that could underlie this relationship. Firstly, persistent viral infections can cause long-standing gut pathology, as the viral infection itself causes extensive mucosal damage. Secondly, the aberrant immune response in immunodeficient patients frequently results in hyperinflammation. It is possible that this hyperinflammation results from overproduction of proinflammatory cytokines, aimed at directing the (defective) immune system towards the site of infection. Thirdly, we speculate that

classical autoimmunity might occur, resulting from either defective immune regulation (as part of CVID) or as the result of abnormal presentation of autoantigens.

HPeV and norovirus infections usually cause mild symptoms in healthy individuals and rarely lead to serious complications. The patient described here had a long-standing infection with both viruses, which were present for at least 2 years before occurrence of the PLE. Over the years, he has been compliant with IVIG therapy and the manufacturer of his IVIG has not changed; however, we cannot rule out the possibility that certain batches contain lower levels of antibodies to HPeV. Data on the levels of specific antibodies in IVIG and their level of protectiveness is not yet available. We hypothesize that this chronically active infection enabled the development of autoimmune enteropathy in this patient. The finding of large numbers of T cells in the entire gastrointestinal tract supports this hypothesis, although we could not confirm whether these T cells were directed against HPeV. Although seemingly contraindicated, the use of immunosuppressive therapy usually results in marked clinical improvement in immunodeficient patients with symptoms of autoimmune disease.

Eradication of viral infections in agammaglobulinemic patients is important not only for viral symptom relief, but also for the prevention of immune dysregulation. Persistent infections in immunocompromised hosts may also imply public health issues. Patients with persistent excretion of, for example, norovirus or HPeV pose a permanent risk of outbreaks in their environment, as soon as strict hygiene is not practiced. In the case of persistent poliovirus excretion, chronic carriers may eventually even preclude the successful eradication of this disease.[16;17]

Unfortunately, effective therapy is often lacking. Several therapeutic regimens aimed at viral eradication were evaluated in this patient, all failing to influence viral replication. Eventually, pleconaril was synthesized and administered, as this drug had been successful in the treatment of enterovirus/ picornavirus infection in earlier reports.[12;13] Although we were able to synthesize the drug, perform drug purity assays and maintain trough levels within the therapeutic window, we observed no effect on HPeV excretion. This might be due to lack of susceptibility, the concurrent immunosuppressants or the fact that the underlying immunodeficiency precludes viral eradication, irrespective of the antiviral drugs used. Preliminary *in vitro* data showed that the HPeV strain of our patient was not susceptible to pleconaril (data not shown). Correction of the immunodeficiency, with cellular therapies or by stem cell transplantation, could be an alternative strategy to achieve viral eradication; the improved safety of these strategies might certainly enable their use in this patient category.

For patients with hypogammaglobulinemia, future studies should include the role of gastrointestinal infections towards enteropathy. As enteropathy is an important complication in CVID,[18] screening for enteropathogenic viruses could be of immense value. Furthermore, there is a great need for improved safety of more definitive methods to correct the underlying immunodeficiencies in these patients.

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THE ROLE OF PROLONGED VIRAL GASTROINTESTINAL INFECTIONS IN THE DEVELOPMENT OF IMMUNODEFICIENCY RELATED ENTEROPATHY

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Submitted

ABSTRACT

Patients with primary immunodeficiencies are prone to develop enteropathy of unknown pathogenesis. We hypothesize that ineffective clearance of gastrointestinal pathogens, particularly viruses, in combination with defective immune regulation may cause inflammatory enteropathy in certain immunodeficient hosts. We reviewed publications related to prolonged enteric viral infection, immunodeficiency and the subsequent development of inflammatory enteropathy. Prolonged infection with especially enteroviral infections was reported more often in immunocompromised hosts than in healthy individuals. Protracted enteric viral shedding was not always associated with the presence or duration of gastrointestinal symptoms. The development of immunodeficiency associated enteropathy after prolonged viral infections was described in sporadic cases. Clinical consequences of viral gut infections in immunocompromised hosts comprise isolation issues and supportive care. Prospective studies in cohorts of immunodeficient patients are required to study the impact of prolonged enteric viral replication with respect to the pathogenesis of non-infectious enteropathy.

INTRODUCTION

Enteropathy involves pathologic changes in the intestine, commonly associated with inflammation, and is typically symptomatic.[1] The most common non-infectious enteropathies are inflammatory bowel diseases (IBD), of which the two major forms are ulcerative colitis and Crohn's disease. Other entities include autoimmune enteropathy, gluten-sensitive enteropathy or celiac disease, idiopathic (protein-losing) enteropathies and syndrome-associated enteropathy, as observed in common variable immunodeficiency (CVID).

IBD is generally believed to result from a disturbed balance between mucosal immunity and the luminal microflora. Many components of the mucosal immune system have been implicated to contribute to IBD pathogenesis. Alterations in the first barriers, i.e. intestinal epithelium and innate immune system, consist of disease-associated genetic polymorphisms involved in e.g. autophagy.[2-6] Second, defects in acquired immunity may contribute to the disease. Described perturbations in adaptive immunity include aberrant $CD4^+$ T helper cell responses,[7] and susceptibility variants in the IL-23 receptor gene.[8;9] Autoantibodies and increased numbers of B cells were found in IBD.[10;11] These exaggerated adaptive immune responses may result in perpetual inflammation and intestinal damage.

Regarding microbial triggers, research is mostly aimed towards bacteria, but viruses may be involved as well.[12] Cadwell *et al*. show in mice with the Crohn's disease susceptibility gene *Atg16L1* that a specific persistent murine norovirus infection induces Paneth cell abnormalities. When subsequent intestinal injury was induced, these mice developed a severe inflammation resembling Crohn's disease. Intestinal injury remained self-limiting in wildtype mice and *Atg16L1* mice without a persistent norovirus infection. The authors conclude that a virus-plus-susceptibility interaction, in combination with environmental factors and commensal bacteria, can determine the phenotype of hosts carrying common risk alleles for inflammatory disease.[13]

These findings suggest that common viruses may also initiate enteropathy in immunologically susceptible humans. Patients with primary immunodeficiencies (PID) are particularly prone to develop enteropathy.[14-16] IBD affects around 0.3% of the individuals in northern developed countries,[17-20] while the estimated prevalence of enteropathy in PID patients is higher; e.g. 10% in CVID patients.[15] This immunodeficiency associated enteropathy causes significant morbidity and mortality, and may necessitate immunosuppressive treatment.[21] PID patients frequently suffer from parasitic and bacterial gastrointestinal infections,[22;23] but regularly, gastrointestinal symptoms occur without the detection of a pathogen when using conventional screening methods. Recently, the detection of viruses has been facilitated by the development of molecular techniques (reverse transcription polymerase chain reaction, RT-PCR), showing superior sensitivity when compared to viral cultures.[24] Patients with a defective cellular immunity, such as individuals with severe combined immunodeficiency (SCID), are evidently prone to viral infections, but the rate of infection is also enhanced in patients with antibody deficiencies. In some patients, these viruses may persist for several years.[25;26]

Taken together, we considered the possibility that inflammatory enteropathy in immunodeficiency can be triggered by common gastrointestinal virus infections. The continuous replication of a virus may cause intestinal changes, augmented by a persistent but aberrant immune response, leaving the gut in a perpetuating inflammatory state.[26] We reviewed the literature to define the prevalence of chronic common gastrointestinal virus shedding, in both immunodeficient and immunocompetent individuals. Also, we explored the potential association between these viruses and the development of enteropathy.

METHODS

We systematically searched the MEDLINE database up to April 2011 for relevant peer-reviewed papers. Search details are depicted in figure 1. Search terms were designed to find articles concerning prolonged viral shedding, the relation between common enteric viruses and enteropathy, and the relation between common enteric viruses and immunodeficiency. Titles and subsequently abstracts were screened by two authors independently (AvdV and DHvK) based on predefined inclusion and exclusion criteria. Available full text papers were assessed for relevance and validity, and related articles and reference lists of relevant papers were screened.

RESULTS

Ninety papers were reviewed of which 53 are summarized in table 1. The occurrence of prolonged viral shedding is summarized per enteric virus for healthy and immunocompromised hosts; if data were available, the association with enteropathy is described.

Norovirus

Norovirus infection is with 5-36% the major culprit of gastroenteritis epidemics among adults and children.[27;28] After challenging healthy individuals, fecal shedding of norovirus persisted for up to 2 weeks, especially in volunteers that developed symptoms.[29] Over 90% of individuals cleared norovirus within 3 weeks, although occasionally, fecal shedding persisted for up to 5 weeks.[30] Other studies in immunocompetent individuals showed a range of 13-56 days.[31] Viral shedding was not associated with clinical symptoms. In physiologically more susceptible children and elderly, fecal shedding ranged from 9 to 47 days, irrespective of clinical symptoms.[32-34]

In immunodeficient individuals, viral shedding may be prolonged.[35-38] The median norovirus excretion was 16.5 and 28 days in immunosuppressed adults and children, respectively, compared to 4 days in previously healthy hospitalized patients.[39] In other immunocompromised patients, norovirus excretion lasted for a median of 13 weeks.[30] Pediatric oncology patients shedded norovirus for up to 140[40] or 433[41] days during

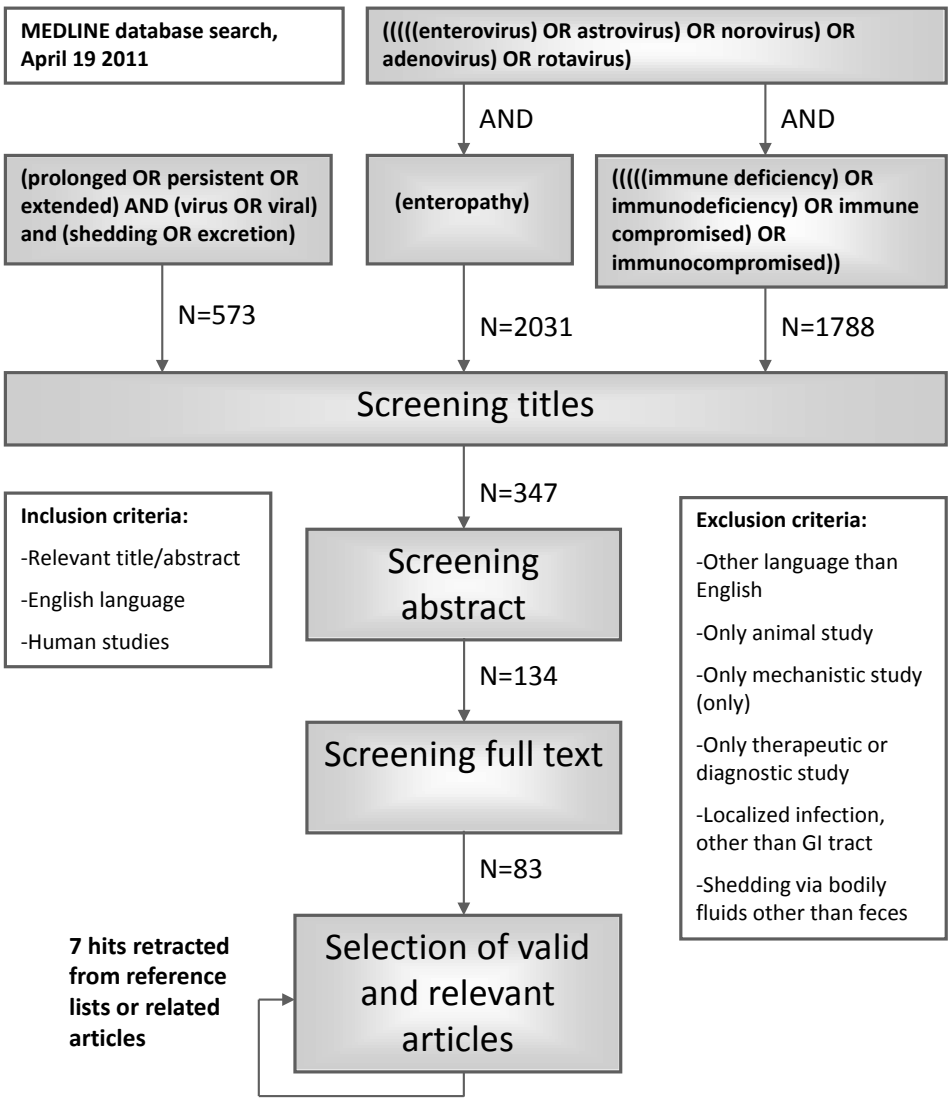

Figure 1: Flow chart of literature selection procedure.

an outbreak. The duration of shedding and presence of clinical symptoms were not always associated.

We identified one paper on the association of norovirus with enteropathy, describing exacerbations of inflammatory bowel disease associated with norovirus infections in children.[42]

Table 1: Literature on enteric virus shedding.

Reference	Study population	Number	Diagnostic method
Norovirus			
Okhuyen *et al.*[29]	Healthy individuals	72	ELISA
Gallimore *et al.*[36]	Child with T-cell def, following BMT	1	RT-PCR
Simon *et al.*[40]	Pediatric oncology patients	20	RT-PCR
Murata *et al.*[33]	Children,18 mn (3 mn - 7 y)	71	RT-PCR
Atmar *et al.*[31]	Healthy adults (18-50 y)	16	RT-PCR
Lee *et al.*[37]	10 mn old, multiple organ transplant recipient	1	RT-PCR
Ludwig *et al.*[41]	Pediatric oncology patients on immunosuppressants, 1.8 y [0.5-15.8]	9	RT-PCR
Siebenga *et al.*[30]	Patients in tertiary care with norovirus infection; focus on 8 pt with shedding>3 wk, aged < 3 y (n=5), 37, 67 and 69 y	8[d]	RT-PCR
Tu *et al.*[34]	Patients in an age-care facility, 85 y (58-93)	14	RT-PCR
Henke-Gendo *et al.*[39]	Hospitalized subjects, 64 y [14 d-89 y], 20 immunocompromised	75	RT-PCR
Aoki *et al.*[32]	Patients in an age-care facility (60-98 y)	13	RT-PCR
Wingfield *et al.*[35]	36 y HIV+ with poor therapy compliance	1	PCR
Schwartz *et al.*[38]	Immunosuppressed patients (chemotherapy or HSCT, 17-71 y) and staff members (20-56 y)	26 patients 11 staff members	RT-PCR
Rotavirus			
Saulsbury *et al.*[50]	Children with PID[e] 4 mn-18 y; controls < 3 y hospitalized for GE; healthy controls 6 mn-16y	23 pts, 46 ill controls, 39 healthy controls	ELISA
Mata *et al.*[48]	Children in rural Guatemala	45	ELISA
Wilde *et al.*[52]	Children <2 y in intermediate care[f]	9	RT-PCR and ELISA
Gilger *et al.*[53]	Children with SCID, DiGeorge or AIDS 4-8 mn	4	ELISA or EM
Richardson *et al.*[47]	children admitted for severe rotavirus diarrhea, 1-39 mn	37	RT-PCR and EIA
Cunliffe *et al.*[45]	In- and outpatient children, HIV+ and HIV-, median age 8-9 mn [1-55]	29/102 HIV +; 45/348 HIV- [h]	EIA
Mori *et al.*[54]	female 87 y with impaired NK cell activity	1	Rota-Kit
Rodriguez-Baez *et al.*[49]	Hospitalized children 0-6 y	309	ELISA
Rayani *et al.*[46]	Pediatric cancer patients; Rota + or -;	28 pts, 28 controls	ELISA

Duration symptoms, median [range]	Duration viral shedding, median [range]	Association symptoms and shedding
N/A	peak 72 h, up to 2 wk	partly[a]
156 d	156 d	
7 d [1-140]	23 d [3-140]	
< 2 y: 7 d; 2-5 y: 3,5 d	16 d [5-47]	
1 d [10-61 h]	28 d [13-56]	no[b]
Chronic diarrhea, duration not defined	114 d	
19 d [7-433]	46-64 d[c] [32-433]	no
N/A	Immunocompetent: 14 d [7-35] Immunosuppressed: 91 d [21-182]	
3 d [1-4]	28.5 d [13.5-44.5]	no
N/A	In healthy 4 d [1-61]; in immunosupressed 16.5 d [1-53]; immunosupressed children 28 d [1-53]	no
3 d [1-6]	13 d [9-32]	no
Chronic diarrhea >6 mn	481 d	
Patients 7 d [2-36]; staff 3 d [1-13]	Patients 36 d [9-118]	
2 pts diarrhea > 6 wk, 2 pts with acute self-limiting GE < 12 d	2 immunodeficients > 6 wk, 2 immunodeficients 10-12 d, 8 ill controls with no excretion at follow-up (2 - 12 d)	yes
N/A	1-5 wk (80% <1 wk)	
N/A	9.5 d [1-19] RT-PCR; 5.7 d [1-17] ELISA	no
All had chronic rotavirus diarrhea until death	5.25 wk [2-8]	
4-5 d (mean), range 1-14 d diarrhea	10 d [4-57] by RT-PCR; 7 d [4-29] by EIA[g]	no
2-3 d [0-12] diarrhea (HIV+/- similar)	6/29 HIV+ shed virus: 2 wk [1-3 wk]; 2/45 HIV - shed virus: 1.5 wk [1-3 wk]	no
3 d	min 34 d[i]	
N/A	Max 7 d	yes
7 d [4-34]	17 d [4-73]	

Table 1: Continued.

Reference	Study population	Number	Diagnostic method
Ramani *et al.*[51]	Neonates	103 (33 with symptoms)	RT-PCR
Enterovirus I; poliovirus			
Alexander *et al.*[70]	review human polio excretion 1935-1995	373 WT polio, 1900 VAPV	Variable cell cultures
Kew *et al.*[74]	16 y old male with CVID	1	Cell Culture
Bellmunt *et al.*[73]	CVID male, 7 y	1	Cell culture and RT-PCR
Martin *et al.*[75]	20 y old female with hypogammaglobulinemia	1	Cell culture
Fiore *et al.*[78]	Patients with XLA, 14.5 y [3-33]	38	Cell culture
Halsey *et al.*[77]	XLA, CVID or IgAdef patients, 6.8-26.7 y	306 IgG; 40 IgA deficient	Cell Culture and PCR
MacLennan *et al.*[25]	24 y male, CVID	1	Cell Culture
Martin *et al.*[71]	healthy boy 1 y, vaccinated with OPV	1	Cell culture and RT-PCR
Hennessey *et al.*[67]	HIV+ adults 26-35 y [18-65]	419	Cell culture
Yang *et al.*[76]	Boy 8 y, diagnosed with CVID	1	Cell culture
Asturias *et al.*[80]	HIV-infected children (3.6 y [1.5-15]) and adults (25.2 y) and children with cancer and > 1 y of chemotherapy (8.7 y [2-16]); all OPV	94; 101; 50	Viral culture
Pavlov *et al.*[68, 69]	Immunodeficient children (mostly HIV+), aged 4 mn-8 y	164	RT-PCR
Enterovirus 2; non polioviruses			
Bailly *et al.*[84]	SCID girl, 7 y, associated with CHH syndrome	1	Cell culture
Johnson *et al.*[85]	3.5 mn old infant with agammaglobulinemia	1	ELISA
Liste *et al.*[86]	HIV+ children, 1-60 mn	6	RT-PCR
Chung *et al.*[65]	Hospitalized children with enterovirus, 1 mn - 5 y	12	Cell Culture and IF
Gouandjika-Vasilache *et al.*[81]	HIV+ adults, mean age, 32.5 y;	28	Cell Culture
Cheng *et al.*[64]	A term neonate (symtoms 48 h after birth)	1	RT-PCR

Duration symptoms, median [range]	Duration viral shedding, median [range]	Association symptoms and shedding
0-4 d	Entire follow-up period [35-75 d]	no
110 asymptomatic infections, 263 paralytic or meningitic poliomyelitis patients	3-4 wk in many persons; up to 5-6 wk in a smaller no. of persons	
Paralytic poliomyelitis at age 16 y	189 d confirmed, (est: 6.9-9.3 y)	
Poliomyelitis anterior onset at age 7	5.5 y[j]	
Patient was fed monotypic Sabin 3 vaccine, no symptoms	+/- 21 mn (631 d)	
N/A	No excretion	
N/A	No long-term excretion found	
None	9 y confirmed, (est: 22 y)	
None	4 mn confirmed, (est: 10 mn)	
N/A	No excretion	
Bulbospinal poliomyelitis onset at age 8 y	10 mn[k]	
N/A	No excretion	
The children were hospitalized for various reasons	Polio detected in 13 patients: period unknown, person to person transmission not excluded	
not shown	22 mn	
Admitted for pneumonia, diarrhea, failure to thrive	>70 d	
N/A	1.6 to 6 mn	
Different symptoms, associated with EV infection	3-11 wk (mostly 4-8 wk)	
N/A	No polio excretion detected; other enteroviruses in only 1 sample	
13 d	23 d (after ilness onset)	

Table 1: Continued.

Reference	Study population	Number	Diagnostic method
Astrovirus			
Noel *et al.*[95]	2 SCID patients	2	RT-PCR
Mitchell *et al.*[91]	Children attending day care centres [2- 30 mn]	36	EIA vs RT-PCR
Maldonado *et al.*[92]	Rural Mayan birth cohort	271 infants	ELISA (confirmed by RT-PCR)
Gallimore *et al.*[90, 94]	- 8 mn SCID patient with BMT - 10 mn old IPEX patient with HSCT	1	RT-PCR
Adenovirus			
Van *et al.*[101]	Healthy children in day-care, mean 13 mn	94	EIA
Munoz *et al.*[103]	Hospital records of patients with positive adenovirus cultures	440	Cell culture
Lee[37]	10 mn old, multiple organ transplant recipient	1	RT-PCR
Curlin *et al.*[104]	HIV positive males	20	RT-PCR
Other viruses			
Picobirna Grohmann *et al.*[117]	HIV positives	1	PAGE
Picobirna Gallimore *et al.*[116]	Healthy adult	1	PAGE
Caliciviruses Rockx *et al.*[114]	Community based cohort	99	RT-PCR

[a] Prolonged shedding in >90% of ill, 50% of asymptomatics. [b] Only symptomatic participants included; no difference between participants who met predefined criteria or those who did not. [c] Last positive 46 d; first negative 64 d. [d] This study found 131 patients norovirus positive; of those 131, 8 patients (8,4%) excreted NoV for ≥3 wk and were included. [e] 1 SCID, 8 CVID, 3 XLA, 4 IgA deficiency, 3 ataxia telangiectasia, 2 transient hypgogammaglobulinemia, 2 X-linked hyper IgM syndrome. [f] Of the 40 children, the 9 that were studied >5 d were aged 1 wk to8 months. [g] 30% excreted >20 d. [h] 1186 children studied, 450 had rotavirus infection (102+348); of these 74 (29+45) completed follow-up. [i] First positive sample March 5; last positive April 10, first negative April 26. [j] 5.5 y confirmed; phylogenetic data suggested an additional 4-6 y of unrecognised poliovirus shedding before onset of paralyis. [k] 10 months of excretion confirmed; 30-35 months estimated. [l] Virus excreted longer in symptomatic children (4.2+ 0.4 d vs. 2.8+ 0,5 d, p=0.04). [m] No samples from later timepoints examined, shedding could have continued longer. Newborns more likely to shed virus >15 d. ELISA=enzyme-linked immunosorbent assay; N/A=not assessed; RT-PCR=reverse transcriptase polymerase chain reaction; HIV=human immunodeficiency virus; HSCT=hematopoietic stem cell transplantation; PID=primary immunodeficiency; GE=gastroenteritis; SCID=severe combined immunodeficiency; AIDS=acquired immunodeficiency syndrome; NK cell=natural killer cell; CVID=common variable immunodeficiency; XLA=X-linked agamamglobulinemia; OPV=oral polio vaccine; CHH=cartilage-hair hypoplasia; BMT=bone marrow transplantation; IPEX=immunodeficiency polyendocrinopathy, enteropathy, X-linked syndrome.

Duration symptoms, median [range]	Duration viral shedding, median [range]	Association symptoms and shedding
N/A	32d and 102 d	
59% had diarrhea 7.5 d [1-23]	EIA: 1.5 d [1-9] RT-PCR: 4 d [1-35]	
26% with astrovirus had symptoms	61% had astrovirus; of those, 70% was (intermittently) positive for 5 wk [0-17]	
Diarrhea, duration unknown	156 d Around 120 d	
46% asymptomatic	Mean 3.9 d [1-14] In immunocompromised patients 26.6 d [24-33]	yes[l]
Chronic diarrhea, duration unknown	114 d	
	4 [1-9]	
chronic diarrhea, 7 mn	7 mn	
1 d mild diarrhea	107 d	
5 d	26% up to 22 d[m]	no

Rotavirus

Rotavirus infection may cause severe gastroenteritis amongst children worldwide, resulting in 500,000 deaths a year.[43;44] Symptoms associated with rotavirus disease usually last for 2-7 days.[45-48] The occurrence of symptoms was in some[49;50] but not all[47;51] studies related to rotavirus shedding.

Several studies have shown normal shedding periods of approximately 10 days in healthy children, while adults have not been described.[45;47;52] Extended fecal shedding occurred sporadically,[47] but could persist in neonates for up to 75 days, albeit with low copy numbers of virus.[51]

In immunocompromised hosts, prolonged fecal shedding has been documented more often and gastrointestinal symptoms may persist for weeks.[46;50;53] Rotavirus shedding was present for at least 34 days in an 87-year-old female with impaired natural killer (NK) cell activity.[54] RotaTeq vaccine-derived rotavirus strains were shed for 1 to 12 months by SCID patients.[55-57] In human immunodeficiency virus (HIV)

positive, oncology or SCID patients, excretion persisted for more than 2[45;46] to over 6 weeks.[45;50;53;58]

Rotaviruses were linked to enteropathic diseases in a prospective study which showed that recurrent rotavirus infections increased the risk of developing celiac disease in genetically predisposed children.[59] In active celiac disease, but not in patients on a gluten-free diet, a subset of anti-transglutaminase IgA antibodies recognized rotavirus major neutralizing protein VP-7, suggesting possible involvement of rotavirus infection in the pathogenesis of the disease.[60] Additionally, two patients developed celiac disease following rotavirus infection, as demonstrated by an increase in anti-rotavirus antibodies that decreased during follow-up.[61] No differences were found in serum rotavirus-antibody levels between IBD patients and controls.[62] Finally, the report of a patient with protein-losing enteropathy after a Fontan surgery procedure suggested rotavirus to trigger this complication.[63]

Enterovirus

Gastroenteritis due to enteroviruses is common, but severe and disseminated disease is usually restricted to young infants.[53;64;65] Several studies have investigated the duration of enterovirus excretion, most notably poliovirus. Vaccination program strategies have eradicated polio in several continents;[66] although due to the use of live oral poliovirus vaccine, and the fact that sporadic immunocompromised patients fail to clear the infection and thus become chronic excretors, vaccine-derived poliovirus (VDPV) still circulates in the population.[67-69] VDPV may be excreted up to 4-8 weeks by a large proportion of susceptible healthy individuals;[65;70] one healthy infant excreted VDPV for 10 months.[71]

Extended VDPV excretion particularly occurs in antibody deficiency syndromes,[72] ranging from 21 months to 22 years in CVID and related hypogammaglobulinemias.[25;73-76] Nineteen individuals with poliovirus excretion for >6 months are known to the World Health Organization.[77] Nonetheless, others did not find prolonged VDPV shedding in a cohort of 38 X-linked agammaglobulinemia (XLA) patients,[78] 16 PID patients[79] and 346 IgG or IgA deficient patients,[77] suggesting that these chronic carriers are incidental cases. In HIV-positive individuals, who predominantly have T cell defects but may develop humoral deficits, prolonged VDPV shedding (>6 months) has not been reported,[67-69;80-82] except for one study in which person-to-person transmission however could not be excluded.[68;69]

Data on prolonged shedding of other enteroviruses is scarce and limited to immunocompromised hosts.[83] Coxsackievirus shedding persisted for 10 more days after symptoms had subsided in a healthy neonate,[64] while enteroviral excretion in immunodeficient patients ranged from 70 days to 22 months.[84-86] A patient with secondary antibody deficiency developed meningoencephalitis due to echovirus 13.[87] Chronic meningoencephalitis was seen in XLA; here, an echovirus type 11 persisted for 7 years.[88]

An association between long-term VDPV excretion and enteropathy has not been reported. However, one XLA patient developed enteropathy resembling Crohn's disease with enterovirus in the inflamed intestinal tissue and adjacent mesenteric

lymph nodes, but not in biopsies of the surrounding unaffected ileocolonic tissue. This case illustrates the possibility of a relation between enterovirus and enteropathy in hypogammaglobulinemia.[89]

Astrovirus

Astrovirus causes acute viral enteritis;[66;90] in healthy children, shedding usually lasted several days, but could persist for up to 35 days, often asymptomatically.[91] Results from a birth cohort of rural Mayan infants were very different from Western study cohorts; 61% was astrovirus positive during the 18-week study period. Seventy percent of them were positive for a median of 5 weeks, although patients that were intermittently positive were not excluded. Only one-quarter suffered from clinical symptoms.[92]

Astrovirus infections are frequent in immunocompromised patients.[66;90;93] During an outbreak in a pediatric PID unit, two patients excreted astrovirus for 156 days.[90;94] Two SCID patients excreted astrovirus for 32 and 102 days.[95] Astrovirus infections did not occur more frequently in HIV-positive individuals.[96;97] No relation between astrovirus infection and enteropathy has been reported, excepting a case report that associates astrovirus infection with the onset of celiac disease.[98]

Adenovirus

Adenovirus gastroenteritis occurs mostly in young children[99] and in immunodeficient populations.[93;100] In healthy children, Van *et al.* showed that fecal shedding of adenovirus occurred for a mean period of 3.9 days (range 1-14). Adenovirus excretion was associated with symptoms in half of the infected population and persisted longer in symptomatic children.[101] In one-third of another previously healthy pediatric population, diarrhea remained for over 2 weeks.[99] One study found prolonged viral shedding in 11 healthy infants for up to 515 days.[102]

In the immunocompromised, adenovirus shedding was found to continue longer than in previously healthy children (22 vs. 8 days, respectively).[103] Adenovirus shedding was reported for 114 days in a 10-month-old multiple organ transplant recipient, and accompanied by norovirus shedding and chronic diarrhea.[37] Curlin *et al.* detected adenovirus in 75% of the HIV-positive males that underwent an intensive screening protocol for 18 weeks. Multiple recurrent adenoviral infections were found, lasting for a median of 4 days [1-9].[104]

Adenovirus was implicated in celiac disease pathogenesis as homology was found between the early region Eb1 protein of human adenovirus serotype 12 and A-gliadin, the component activating celiac disease.[105] Neutralizing antibodies to adenovirus 12 were found in 80% of untreated celiac patients, compared to 33% and 31% in treated adults and children, respectively, and 0% to 13% healthy controls.[106] Conversely, studies on the presence of adenovirus 12 DNA on duodenal biopsy samples of celiac disease patients found no evidence for a role in the pathogenesis of celiac disease.[107;108] Second, adenovirus may play a role in HIV enteropathy,[109] defined as enteropathy in HIV-positive patients after exclusion of other pathogens.[110-112] Thomas *et al.* found evidence for adenovirus colitis in 4 of 13 HIV-positive patients with adenovirus present in the stool, and suggested that a proportion of

HIV-positive patients with an enteric adenovirus infection may develop overt mucosal inflammation and chronic diarrhea.[113]

Prolonged fecal excretion of other viruses

Although beyond the scope of this review, prolonged fecal excretion has been reported for several other viruses. Caliciviruses other than norovirus may be excreted in the feces up to 22 days in 26% of the cases, especially in infants.[114] Sapovirus is usually cleared within 2 weeks; some individuals however excreted the virus in high titers for 2-4 weeks.[115] Picobirnavirus was excreted for 107 days in an immunocompetent person without symptoms[116] and for 7 months in a HIV-infected person with chronic diarrhea.[117] Lastly, when plasma viral loads are high, non-enteric viruses can also be excreted in the feces; hepatitis E virus for example was excreted in the stool for 52 days[118] and influenza virus for >2 months in the stool of an immunocompromised child.[119]

DISCUSSION

The purpose of this review was to search the scientific literature on prolonged viral infections in the gastrointestinal tract and the possibility of a relation with the occurrence of immunodeficiency related enteropathy. We found that prolonged carriership of several viruses, in particular enteroviruses, was described more often in immunodeficient patients than in healthy persons. Several reports describe exacerbations of non-infectious enteropathy triggered by viral infections. These findings lend support to further explore the potential relation between chronic carriership of gastrointestinal viruses and the occurrence of non-infectious enteropathy in patients with primary immunodeficiencies.

Several reasons preclude a formal meta-analysis of the data provided in the literature. First, the majority of studies were retrospective, had small sample sizes, included various types of immunodeficiencies, and used different definitions to describe for example prolonged shedding of viruses in stool. Second, sample selection bias is inevitable, since primary immunodeficiency patients are monitored vigorously for viral infections. In many studies, the duration of viral shedding was not associated with the presence or duration of gastrointestinal symptoms; asymptomatic prolonged shedding may thus be missed, especially in the healthy population. Third, prolonged viral shedding could not always be clearly distinguished from re-infections. As a result, it is to date not feasible to draw conclusions at the level of patient groups or in the general population.

Nonetheless, several interesting observations were made. Table 2 suggests that prolonged shedding occurs more frequently in immunocompromised individuals, although these figures were not weighted for proportions of affected individuals. The susceptibility for prolonged carriership seems dependent on the virus genus and on the type of immunodeficiency; in general, immunodeficient individuals appear particularly susceptible to noro- and enteroviruses, but not to rotavirus. Norovirus infections depend on genetic susceptibility factors in the host; blood group O individuals are

Table 2: Overview of the duration of enteric virus shedding per population.

	Range of shedding duration (days) in humans				
	Immunocompetent		Immunocompromised		
Virus	**Adult**	**Infants, elderly**	**B cell**	**Combined**	**T cell**
Norovirus	1-61	5-47	N/A	1-433	156; 481
Rotavirus	N/A	1-75	10-73	10-73	7-56
Enterovirus 1; poliovirus	21-42	0 -120	0-3285	no long-term excretors	no long-term (>180d)
Enterovirus 2; non polio	N/A	77	> 70	660	48-180
Astrovirus	N/A	1-35	N/A	32; 102; 120; 156	N/A
Adenovirus	N/A	1-14, up to 515	24-114	24-114	1-9

N/A=not assessed

more susceptible, while individuals, homozygous for attenuating mutations in the *FUT2* gene (encoding α(1,2)fucosyltransferase) and thus not expresssing the H type-1 oligosaccharide ligand required for Norwalk virus binding, were not infected upon challenge.[120] It remains unclear whether this concerns short- or long-term immunity, and additional factors are probably involved. The lengthy enteroviral shedding in hypogammaglobulinemic patients supports the notion that its intestinal clearance depends on humoral immunity. Several classes of immunoglobulins are involved in antiviral immunity.[121] B1 B cells produce 'natural' polyreactive IgM antibodies[122] which protect the host by e.g. recruiting antigen into germinal centers. Here, in conjunction with $CD4^+$ T cells, naive B cells develop into long-lived plasma cells or memory B cells, both responsible for long-term secretion of neutralizing specific antibodies. Particularly dimeric secretory IgA antibodies are essential, as they are transported across the intestinal epithelium.[121] HIV-related $CD4^+$ T cell lymphopenia in infancy precludes the generation of those long-lived virus-specific memory B cells, while with HIV infection later in life, these memory B cells have already been formed and can survive without the presence of T helper cells.[123] Interestingly, HIV-positive patients did not demonstrate prolonged shedding, except for one study investigating HIV-seropositive infants.[86]

SCID patients, expressing a combined T- and B cell immunodeficiency, showed prolonged fecal shedding and symptomatic phases of gastrointestinal viruses. These infections thus contribute to the patient's inherent failure to thrive. In contrast, patients with humoral immunodeficiencies can become chronic excretors but are usually asymptomatic. We hypothesize that in humoral immunedeficiencies, disturbed specific antibody formation precludes final clearance of the virus, but T cell immunity prevents a severe symptomatic course.

Literature on a possible association between gastrointestinal virus infections and the development of enteropathy was limited. Several studies suggested a link between certain viruses and enteropathy (*table 3*). As these observations were made in singular patients,[63;89;98] or contradicted by findings of other research groups,[62;107;108]

Table 3: Associations of enteric viruses with enteropathy.

Virus	Type of enteropathy	Reference
Norovirus	Inflammatory bowel disease exacerbations	(41)
Rotavirus	Celiac disease	(57-59)
	Protein-losing enteropathy after Fontan surgery	(61)
Enterovirus	Crohn-like enteropathy in X-linked agammaglobulinemia	(87)
Astrovirus	Celiac disease	(96)
Adenovirus	Celiac disease	(103;104)
	HIV or AIDS enteropathy	(111)

the relation remains uncertain. Apart from the presence or absence of prolonged viral infections, other factors may play a role in the development of enteropathy, including factors that control the regulation of the immune system.

The clinical consequences of these findings at the moment include the following aspects. Patients with combined immunodeficiency should be screened at regular intervals for viral carriership in the gastrointestinal tract using RT-PCR, as this is the most sensitive method. Findings may implicate isolation measures to prevent spreading of viruses. In some cases, attempts to eradicate viral infection can be considered. Furthermore, the presence of viral infections may be of prognostic value in case stem cell transplantation is considered. Patients with humoral immunodeficiency can also be chronic carriers of gastrointestinal viruses, and may thus pose a risk to infect other patients. It remains however unclear what the yield of systematic evaluation by RT-PCR of patients with humoral immunodeficiency would be. We propose including chronic replication of viruses in the differential diagnosis of patients with humoral immunodeficiencies and concurrent enteropathy. We have described a patient that had chronic viral replication and eventually developed enteropathy necessitating the use of immunosuppressive treatment, and it would thus seem logical to consider eradicative treatment in some cases, although treatment options are usually limited.

Taken together, prolonged gastrointestinal virus carriership may occur occasionally, mainly in humoral or combined immunodeficiency. Viral shedding is generally not associated with the presence of clinical symptoms, and limited data linking enteric viruses to the development of enteropathy are available. As a result, prospective long-term cohort studies in PID patients are required in order to establish the prevalence of prolonged viral shedding, as well as to further explore its potential relation with non-infectious enteropathy. Specific PID patients could be screened for genetic variants related to enteropathy- or virus susceptibility. Finally, *in vitro* studies aiming to unravel the interaction between virus and host at the molecular level may be valuable.[124] These studies will contribute to understanding of enteropathy in PID patients and may eventually facilitate preventive therapy in specific patient categories.

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ENTERIC VIRUSES ARE ASSOCIATED WITH INTESTINAL INFLAMMATION IN PEDIATRIC COMMON VARIABLE IMMUNODEFICIENCY AND RELATED ANTIBODY DEFICIENCIES

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ABSTRACT

Introduction: Patients with antibody deficiencies, including common variable immunodeficiency (CVID), suffer from recurrent gastrointestinal infections, and a fraction of these patients develops an enteropathy of poorly understood pathogenesis. We investigated whether chronic gastrointestinal viral infections contribute to the development of CVID-related enteropathy and here report our initial results

Methods: Stool samples from 54 children with CVID or related antibody deficiencies and 52 healthy controls were collected at 0 and 6 months and analyzed for the presence of common enteric viruses using RT-PCR and ELISA. Once a virus was detected, sampling was repeated every 3 months. In addition, questionnaires were filled out and fecal excretion of calprotectin, a surrogate inflammatory marker, was measured.

Results: Children with antibody deficiencies had significantly more gastrointestinal symptoms than healthy children, which were associated with mucosal inflammation in patients but not in healthy controls. The incidence of enteric viruses was higher in the patients (24%) than in controls (12%), although not significantly different (p=0.10). Importantly, the presence of viruses was associated with mucosal inflammation in patients but not in controls. Finally, we found prolonged viral shedding in three patients and one healthy asymptomatic control.

Conclusion: We found an increased incidence of inflammatory gastrointestinal symptoms in CVID, which was associated with the presence of enteric viruses, suggesting a causal relation. Prolonged viral shedding may occur, especially in patients. These findings support the hypothesis that viral infections may trigger (auto) immune-mediated enteropathy in CVID and related disorders.

INTRODUCTION

Antibody deficiencies comprise the largest subgroup of primary immunodeficiencies (PIDs) and are characterized by qualitative with or without quantitative defects in specific antibody production, leading to recurrent infections of mainly the respiratory and gastrointestinal tracts.[1] The most frequent antibody defect is common variable immunodeficiency (CVID), a heterogeneous disorder of hypogammaglobulinemia, recurrent infections and impaired specific antibody production.[2] Although CVID is predominantly a B cell defect,[3-5] less prominent defects have been described in T cells,[6-11] natural killer (NK) cells,[12] invariant NK T cells, [13-15] dendritic cells[16-20] and monocytes. [15;21-26]

Gastrointestinal disease affects 20-60% of the CVID patients[27-30] and can be divided into 4 major forms, consisting of infectious-, autoimmune-, malignant- and CVID-related enteropathy.[31] Enteric infections in humoral immunodeficiency are frequently of bacterial origin such as *Salmonella* and *Campylobacter* species, *Yersinia enterolytica*, *Clostridium difficile*, but also parasitic infestations such as Giardia Lamblia occur often.[27;30] A literature review on viral gut infections in PIDs suggested an increased prevalence and duration of viral infections (Van de Ven *et al.*, submitted). Chronic diarrhea is common in infectious causes and may lead to malabsorption. [27;29;30;32] Autoimmune manifestations include pernicious anemia, hepatitis and primary biliary cirrhosis.[27] Furthermore, CVID patients are prone to develop malignant disease, especially gastric cancer and small-bowel lymphoma.[27;33;34] Possibly, the frequently observed nodular lymphoid hyperplasia[27;35] predisposes to the development of lymphoma, although this remains controversial.[29;36] Lastly, CVID-related enteropathy is a separate cause of gastrointestinal disease that mimics other gastrointestinal diseases such as Crohn's disease, ulcerative colitis or celiac disease.[32;37] Bowel biopsy specimens may display epithelial lymphocytic infiltration, increased apoptosis, villous atrophy and crypt distortion; main histological differences with classical inflammatory bowel disease (IBD) however are the absence of plasma cells and the frequent presence of follicular lymphoid hyperplasia in CVID specimens.[32;38]

A large survey that investigated disease-related complications in CVID demonstrated that enteropathy occurs in up to 9% of CVID patients and has the highest mortality rate of the non-malignant complications, with a relative risk of 4.0.[39]

The pathogenesis of CVID-related enteropathy remains largely unknown. Polymorphisms in *NOD2*, a disease-modifying gene associated with Crohn's disease, were more prevalent in CVID patients with enteropathy,[40] and anti-enterocyte antibodies have been detected.[41;42]

Other types of immune dysregulation in CVID are well-known and include antibody-mediated autoimmune cytopenias, and granulomatous disease that may affect lungs, spleen and kidneys. The etiology of these disorders is poorly understood. The ameliorating effect of immunosuppressive drugs in CVID-related enteropathy suggests a component of excessive immune activation, at least in the perpetuation of the enteropathy.

We recently reported on a CVID patient with severe protein-losing enteropathy due to CVID-related enteropathy. This patient shedded high titers of parechovirus and norovirus via the feces for a period exceeding two years, prior to developing enteropathy.[43] The clearance of several viruses in the gut relies predominantly on specific antibody production, and these viruses may thus cause a state of chronic inflammation. We assume that the continuous replication of these gastrointestinal viruses, together with the patient's incessant suboptimal immune response, eventually disturbed the mucosal immune homeostasis and initiated enteropathy. From a broader perspective, perhaps viral triggers in the immunological susceptible host can initiate PID-related enteropathy in other immunodeficiencies as well.

To test this hypothesis, we commenced a longitudinal observational study, investigating the prevalence and clearance of viral pathogens in children with CVID and related antibody deficiencies. Here, we present the initial findings, which suggest that viruses are more common in CVID than in healthy controls. Moreover, viral infections were associated with signs of mucosal inflammation in CVID patients but not in healthy controls.

METHODS

Patients and controls

Patients and controls (aged 4-18 years) were prospectively included between April 2010 and July 2011. Three patient groups were included: patients with CVID as defined by criteria of the European Society of Immunodeficiencies (ESID),[44] patients with disorders similar to CVID we refer to as 'CVID-like disease',[45-47] and patients with X-linked agammaglobulinemia (XLA) based on the presence of a mutated *BTK* gene. All CVID-like patients had impaired specific antibody synthesis upon vaccination, recurrent infections and low serum titers of at least one immunoglobulin (Ig) or IgG subclass. All patients were on Ig replacement therapy with intravenous or subcutaneous immunoglobulins. As a control group, healthy children were recruited amongst the hospital staff. In most cases, only one child per family participated. If siblings were included, stool samples were collected at different time points to minimize bias due to contamination between siblings. Exclusion criteria in controls were the presence or strong suspicion of immune-mediated diseases (immunodeficiencies, autoimmune disease) or inflammatory bowel diseases (Crohn's disease, ulcerative colitis or celiac disease). The study was approved by the local institutional review board and written informed consent was obtained for all participants.

Study design

We conducted a prospective observational study in patients and controls. Stool samples were collected at T=0 and T=6 months (*figure 1*). These samples were used for detection of viruses and levels of calprotectin, a cytosolic protein of neutrophils that can be used as a surrogate marker for inflammatory conditions. If a virus was detected in the stool at T=0 and/or T=6 months, additional samples were collected

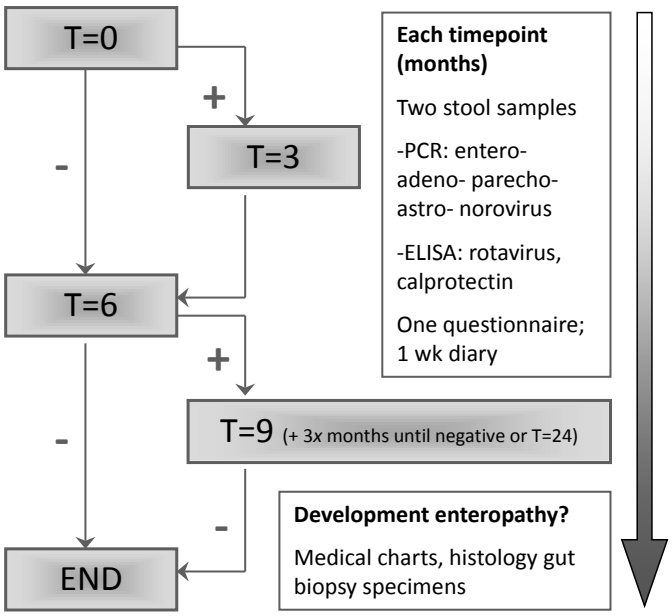

Figure 1: Schematic view of the observational study procedure
PCR=polymerase chain reaction, ELISA=enzyme-linked immunosorbent assay

3 months later to identify long-term carriership (defined as ≥3 months). To evaluate gastrointestinal complaints, all participants filled out questionnaires during the week preceding sample collection regarding defecation frequency, consistency and abdominal pain or cramps.

Enteric virus detection

RNA was extracted from 200 mg stool specimens using 1 mL stool transport and recovery buffer (S.T.A.R.; Roche Diagnostics Corporation, Indianapolis, IN) and 100 µL chloroform. Mixtures were centrifuged and supernatants were diluted with phosphate buffered saline. To monitor for effective extraction and amplification, all samples were spiked with internal control viruses (phocine herpes virus for DNA and murine encephalomyocarditis for RNA). Nucleic acids were extracted with the MagNa Pure LC nucleic isolation system using the total Nucleic Acid Isolation protocol. RNA was reverse transcribed into coding DNA with Multiscribe RT and random hexamer primers (Applied Biosystems). Samples were analyzed by real-time polymerase chain reaction (RT-PCR) in duplicate using PCR Mastermix (Applied Biosystems), and primers and probes specific for the viruses; adenovirus, enterovirus, parechovirus, astrovirus and norovirus (available upon request). Cycling in a Taqman 7500 or 7900 involved the following steps; 2'50°C, 10' 95°C, 45 cycles 15''95°C and 1'60°C; annealing temperature for astrovirus was 53°C. Stool samples were tested for antigens of rotavirus by ELISA using the Oxoid ProSpecT rotavirus microplate assay.

Calprotectin measurements

Calprotectin levels were using a calprotectin ELISA kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's instructions. After an extraction procedure, an antibody to calprotectin heterodimeric and polymeric complexes was coated onto the microplate and extracts were incubated at room temperature for 30 minutes. After washing, a secondary horseradish peroxidase (HRP) conjugated antibody was added and incubated. After another washing step, tetramethylbenzidine was added followed by a stopping reaction. Absorption was measured at 450 nm. Calprotectin levels were divided into 'normal' and 'increased' according to age-related reference values; below 50 µg/g was considered normal for children aged 9 years of older, and less than 166 µg/g normal for children aged 2-9 years.[48]

Statistics

To compare continuous data between two groups, unpaired t-tests were used for parametric data and Mann-Whitney *U* tests for non-parametric data, respectively. Ordinal categorical data were compared using linear-by-linear association; other categorical data were tested with Pearson's chi-square tests or Fischer exact tests if sample sizes were small. Tests were performed two-tailed, and p-values ≤ 0.05 were considered significant. All statistical analyses were performed with SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, U.S.A).

RESULTS

Children with antibody deficiency syndromes display more gastrointestinal symptoms than healthy children

Fifty-four pediatric patients and 52 pediatric controls were included in the study (*table 1*), and all patients provided stool samples at T=0. There was a male preponderance in the patient group, and patients were slightly older than healthy controls (11.1 *vs.* 9.5 years, p=0.03). Questionnaires were filled out completely by 100% of the patients and 96% of the controls. The patient group more often displayed a deviated defecation pattern than the control group; 20% had a decreased and another 20% and increased defecation frequency (p=0.045). Next, patients suffered significantly more days of the week from abdominal discomfort; >50% of the patient population had at least one day per week abdominal ache or cramps, compared to 37% of the controls (p=0.006). Third, fecal consistency was altered in the patient population: 57% experienced loose stools, compared to 27% of the healthy control group (p=0.003). None of the healthy children had watery diarrhea, while this was present in 13% of patients (p=0.013).

To determine whether these differences were attributed to a specific patient subgroup, we compared CVID, CVID-like and XLA patients. Evidently, the XLA groups consisted exclusively of male patients and they were slightly younger (median age CVID 11.7y, CVID-like 11.3y, XLA 9.0y, p>0.05). XLA patients reported significantly

Table 1: Baseline characteristics of patients and healthy donors at timepoint 0.

	Patients	Healthy donors	P-value
Number	54	52	
Male, n (%)	45 (83 %)	25 (48 %)	<0.001
Age, years ± SD	11.1 ± 3.8	9.5 ± 3.8	0.03
Diagnosis, n (%)			
CVID	26 (48 %)		
CVID-like disease	22 (41 %)		
XLA	6 (11 %)		
Defecation frequency, median [range]	8 [0-74]	7.5 [0-18]	
Decreased (0-4 times/wk)	10 (19 %)	5 (10 %)	0.045
Normal (5-15)	33 (61 %)	43 (83 %)	
Increased (15<)	11 (20 %)	4 (8 %)	
Abdominal ache or cramps, n (%)			
Never	26 (49 %)	32 (63 %)	0.006
Once a week	6 (11 %)	12 (24 %)	
Twice a week	5 (9 %)	4 (8 %)	
Three or more	16 (30 %)	3 (6 %)	
Thin stools, n (%)			
Never	23 (43 %)	37 (72 %)	0.003
Once a week	7 (13 %)	3 (6 %)	
Twice a week	4 (8 %)	5 (10 %)	
Three or more	19 (36 %)	6 (12 %)	
Watery diarrhea, n (%)			
Never	46 (87 %)	51 (100 %)	0.016
Once a week	3 (6 %)	0 (0 %)	
Twice a week	1 (2 %)	0 (0 %)	
Three or more	3 (6 %)	0 (0 %)	
Calprotectin, n (%)			
Normal	41 (77 %)	45 (88 %)	0.14
Increased	12 (23 %)	6 (12 %)	
Viruses, n (%)			
Any virus	13 (24 %)	6 (12 %)	0.10
Enterovirus	1 (2 %)	1 (2 %)	
Parechovirus	3 (6 %)	0 (0 %)	
Norovirus	4 (8 %)	1 (2 %)	
Astrovirus	1 (2 %)	0 (0 %)	
Adenovirus	5 (10 %)	3 (6 %)	
Rotavirus	1 (2 %)	1 (2 %)	

SD=standard deviation, CVID=common variable immunodeficiency, XLA=X-linked agammaglobulinemia.

more often thin stools (CVID 50%; CVID-like 44%, XLA 100%; p=0.002) and an increased defecation frequency (CVID 8%; CVID-like 27%; XLA 50%; p=0.039). There were no differences in other gastrointestinal symptoms, levels of calprotectin or the presence of viruses.

Taken together, children with antibody deficiencies have significantly more gastrointestinal complaints than healthy children, and the most frequently affected patients were those diagnosed with XLA.

Mucosal inflammation is associated with gastrointestinal symptoms and the presence of enteric viruses in antibody deficiency, but not in healthy controls

To allow for distinction between organic bowel disease and non-organic disease such as irritable bowel syndrome (IBS), levels of calprotectin were measured in stool. Calprotectin is a cytosolic protein abundantly present in granulocytes, and can be used as a surrogate measurement of intestinal inflammation.[49] Calprotectin levels were increased in a fraction of both the patient and control group (23% *vs.* 12%, p=0.14). Interestingly, there was a significant association between calprotectin levels and diarrhea in the patients (p=0.046) and a trend towards an association with loose stools (p=0.07). Abdominal pain or deviated frequency patterns could not be related to the levels of calprotectin. There were no such associations found in the healthy controls group. These findings suggest that gastrointestinal symptoms in CVID and related antibody deficiencies are not merely functional complaints, but are associated with mucosal inflammation.

Next, we investigated whether symptomatic mucosal inflammation was related to the presence of enteric viruses. In thirteen patients (24%), a virus was detected in the stools, compared to 6 (12%) healthy controls (p=0.10). Of the patients with a virus, 7 had CVID, 5 CVID-like disease and there was one XLA patient. Noteworthy, the incidence of enteric viruses was significantly associated with increased levels of calprotectin in the patients (p=0.002), but not in the healthy control children (p=0.72). Fifty-eight percent of the patients with viral carriership had increased levels of calprotectin in the stools, compared to 13% of the patients without a virus. These findings suggest that enteric viruses have a differential effect in patients with antibody deficiencies compared to healthy controls.

Prolonged viral shedding occurs in patients and a healthy control and does not necessarily cause symptoms

We hypothesized that prolonged viral shedding, due to an inability of effective viral clearance, would eventually lead to enteric abnormalities, and therefore we tested if these viral infections could be prolonged, defined as lasting ≥ 3months.

Sampling was repeated after 3 months for individuals that tested positive for any virus, and for all subjects after 6 months. Currently, follow-up at 6 months has been fulfilled for 28 patients and 43 healthy controls. Although data collection is not yet complete, we see similar results compared to data gathered at T=0: patients have significantly more often abdominal pain or cramps (patients 54%, control 36%, p=0.035), thin stools (patients 46%, control 21%, p=0.004), and diarrhea (patients 25%, control 0%, p=0.002). Again, there were more patients with an enteric virus present (25% *vs.* 12%, p=0.14) or increased calprotectin levels (15% *vs.* 10%), but these differences were not statistically significant. These findings are consistent with prior findings and thus partly confirm findings at T=0.

Of the virus-positive subjects that were re-sampled after 3 months, 15 had effectively cleared the virus. One patient with norovirus withdrew consent. Two XLA patients and

one healthy control had encountered another virus at T=3 months, which was cleared at T=6. In 2 patients and one control however, viral shedding lasted >3 months (*table 2*). In addition, one XLA patient that was re-sampled due to the presence of an adenovirus at T=6, excreted enterovirus for at least 3 months (*table 2*). Prolonged viral shedding could occur in the absence or presence of gastrointestinal symptoms, and was not necessarily accompanied by mucosal inflammation. Two of the chronic carriers also had a concurrent virus or a virus at a different timepoint, suggesting that these patients were in generally more prone to gastrointestinal virus infections.

Taken together, gastrointestinal viral infections may persist for at least 3 months in patients but also in healthy children, and may cause symptoms and mucosal damage but may also remain asymptomatic.

Table 2: Characteristics of individuals with prolonged viral shedding.

Subject	**1**	**2**	**3**	**4**
Diagnosis	CVID; gradually evolved in late onset CID	CVID-like (SAD+low IgA)	XLA	Healthy control
Gender	Female	Male	Male	Male
Age (years)	13	7.5	4.5	11.5
Virus	Adenovirus (and intermittent norovirus)	Norovirus	Enterovirus	Rotavirus
Abdominal ache or cramps?	Yes, often cramps	Currently not, and usually never	Currently not, and usually never	Currently not, and usually never
Thin or watery stools?	Yes, most days of the week thin stools and a few days also watery diarrhea	Currently not, and usually never	Almost always has thin stools, but never diarrhea	Currently not, and usually never
Defecation frequency	Around 2 times a day	Always once a day	Variable, 1 to 4 times a day	Usually 3 times a day
Calprotectin levels (µg/mL)	142 at first and 30 at second measurement, at other timepoints often increased	33; 36	63; 30; 318 with adenovirus infection	30; 30
Other viruses	Intermittent norovirus	No	Adenovirus	No

CVID=common variable immunodeficiency, CID=combined immunodeficiency, SAD=specific antibody deficiency, XLA=X-linked agammaglobulinemia.

DISCUSSION

This is the first observational study on gastrointestinal disease in children with antibody deficiencies and in healthy controls, and initial results show that antibody deficient

patients have significantly more gastrointestinal symptoms than healthy controls. Furthermore, our results show a significant association between viral carriership and signs of inflammation in patients, which was not observed in controls. Viral carriership appeared to be increased in CVID and related disorders. Extended follow-up in this patient cohort will shed further light on the development of PID-related enteropathy and its relation with viral infections.

The strengths of this study include its prospective design, the high return rate of questionnaires and stool samples, and the use of RT-PCR which is ultra sensitive for detection of viral pathogens in stool. The abdominal complaints were assessed by means of a questionnaire of a one-week diary. Additional questions concerning the representativity of the gastrointestinal symptoms during this week revealed that there was little fluctuation in the amount of symptoms. The questionnaire repeated at T=6 gave similar results, thereby rendering coincidental findings due to e.g. seasonal variations or local epidemics implausible. Although there were some differences in baseline characteristics between the patient and control group, it is improbable that these account for the difference in abdominal complaints. First, we believe it is unlikely that a mean age difference of two years will significantly influence the outcome, as symptoms were not related to age within our patient and control group. Second, there was a male predominance in the patient population. Gender is related to the incidence of gastrointestinal disease; peptic ulcer disease is more frequent in men, while gall stones and IBS occur more often in women. The distribution of IBD is similar between sexes.[50] As functional somatic complaints are more common in females in adulthood and childhood,[50;51] the male predominance is more likely to lead to an underestimation of the complaints in CVID and related disorders. Moreover, the association with mucosal inflammation, as measured by increased levels of calprotectin, suggests that these symptoms are not merely functional, at least not in the patient group.

In line with our hypothesis that persisting infections may trigger PID-related enteropathy, we found a significant association between mucosal inflammation and the presence of enteric viruses in the patients, but not in healthy children. These findings suggest that gastrointestinal viruses usually do not cause (sustained) intestinal damage in healthy children, but may affect the intestinal epithelium in antibody deficiency syndromes. Presumably, common gastrointestinal viruses are efficiently cleared after a few days by the healthy immune system. The individual may experience gastrointestinal symptoms, but in general, complete remission is swiftly achieved. Conversely, in the humorally impaired immune system, an antiviral immune response may be initiated, but remains insufficient to eradicate the virus, as the final step to eradicate the virus is deficient. The continuous presence of a gastrointestinal virus, in patients that have other components in their immunodeficiency that render them more susceptible to dysregulation of the immune system, may eventually result in disturbances of the mucosal immune homeostasis. Our findings of prolonged viral shedding support the possibility of this mechanism. Prolonged asymptomatic virus excretion was also noted in one healthy child, but we hypothesize that a second susceptibility factor must be present to result in clinically relevant enteropathy.

Distortion of the immunological balance may be triggered by molecular mimicry of virus particles with autoantigens. Cellular invasion by the virus leads to surface expression of intracellular components. These components may contain autoantigens that resemble viral or microbial peptides, leading to molecular mimicry and initiate classic autoimmunity. Mechanisms of molecular mimicry have already been proposed in the development of other gastrointestinal diseases: celiac disease has been linked to viral epitopes of rotavirus,[52-54] astrovirus[55] and adenovirus.[56;57]

Alternatively, the cellular damage leads to an exaggerated hyperinflammatory response, due to e.g. altered antigen presentation[16;19] or defective regulatory mechanisms[8;58;59] to dampen the immune response. This is especially important in CVID and CVID-like disease, where defects other than in B cells have been described, including T cells, monocytes and dendritic cells. The lower prevalence of enteropathy in XLA, a pure intrinsic B cell defect, suggests that other cell types are co-involved in CVID enteropathy.[31]

Our findings of increased mucosal inflammation in antibody deficiency, associated with the presence of viruses, are in line with our hypothesis that continuous viral replication in the susceptible host may trigger (auto)immune-mediated enteropathy. This is further supported by the notion of prolonged viral shedding in a few patients. Continued follow-up and additional power is required to firmly establish an associated between prolonged viral shedding and mucosal inflammation, eventually leading to CVID-related enteropathy. To this end, the specific virus strains should be determined to allow for distinction between chronic viral infections and re-infection. Alternatively, mucosal damage could also be maintained by any particular virus, and thus frequent intermittent viral infections could also be relevant to disease pathogenesis. It would therefore be of interest to continue documenting incidental viruses, especially since two of the patients with prolonged viral shedding were co-infected with other enteric viruses. Furthermore, other agents could also be implied in the pathogenesis, such as bacterial pathogens, commensal microflora, and chemical agents. Meticulous monitoring of drug use, as well as studying the microbiome of these patients, would therefore enhance the value of this study.

In conclusion, this study reveals an increased incidence of inflammatory gastrointestinal symptoms in CVID compared to healthy children. Furthermore, the presence of common enteric viruses was associated with an increased incidence of biochemical signs of mucosal inflammation. These findings may contribute to the understanding of the development of enteropathy in CVID and related antibody deficiencies.

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3

GENERAL DISCUSSION

GENERAL DISCUSSION

Primary immunodeficiencies (PIDs) are important diseases to study for several reasons. First, they have severe clinical consequences, as patients suffer from significant morbidity and mortality. Considerable improvement can be achieved by implementation of personalized therapeutic options, which requires the clarification of the underlying cause of distinct PID. Second, the study of primary immunodeficiency diseases teaches us valuable lessons on human immunology; monogenetic defects in particular are experiments of nature that educate us on the exact function of specific molecules in humans *in vivo*. The genetic cause of common variable immunodeficiency (CVID) disorders, combined with environmental factors, is at present often still unresolved. CVID syndrome is featured by disturbed specific antibody production and frequently occurring autoimmunity. CVID patients encompass the largest group of PID patients, with heterogeneous cause of disease and prognosis, and represent a patient population in which exploration of cause of disease and outcome is highly desired. This thesis describes investigations we have performed on several aspects of the disease. In this chapter, current findings are discussed and recommendations for future work are provided.

CVID in childhood: are children comparable to adults?

Most of our understanding of CVID and related antibody deficiency syndromes is based on data gathered from adult patients. As we studied a pediatric CVID population in this thesis, we were able to explore potential differences between children and adults with CVID. Evidently, there are differences due to age. As the immune system matures with age, the total number of lymphocytes and the relative number of naive T and B lymphocytes decrease, while memory subsets gradually increase[1;2]; these physiological age-related changes are also seen in pediatric CVID patients. Additionally, pediatric CVID patients exhibit the same type of lymphocyte subset alterations compared with age-matched controls as adult CVID patients, including decreased fractions of immunoglobulin (Ig) class-switched memory B cells and $CD4^+$ T cells, and increased fractions of newly formed B cells (chapter 2). Moreover, we also found decreased numbers of $CD19^+CD27^+IgD^+IgM^+$ non-class-switched memory or marginal zone B cells in children, which has not been reported as a general characteristic in adult CVID but has been described only in a small adult subset.[3] We propose that the decrease of these $CD19^+CD27^+IgD^+IgM^+$ non-class-switched memory or marginal zone B cells may be more pronounced in pediatric CVID, as the relatively immature pediatric immune system depends more heavily on this B cell subset. When we compare our pediatric CVID population to characteristics of adult CVID patients reported in current literature, there are certain other differences. These are the male preponderance in prepubertal CVID, the relatively high number of familial cases and TACI mutations, and the relatively low rate of intrinsic CVID-related complications in our pediatric population (chapter 2, 3, 8). These apparent distinctions may not hold true and be a consequence of our relatively small cohort size (around 70 individuals), and disappear when different pediatric cohorts are pooled. Also, it is not certain whether our findings

in our population in a tertiary care university hospital will apply to the entire pediatric CVID population or only to our cohort, in particular for clinical complications. In general, childhood patients are diagnosed with little delay and directly started on optimal therapy at early age. This could explain our currently low complication rate, even in comparison to other pediatric populations.[4] Nevertheless, also our patients may be more prone to develop abundant complications in the future, as others have reported that disease-related complications are somewhat more frequent in patients with early age of onset.([5] and C. Cunningham-Rundles, CIS Annual Meeting, Chicago IL, U.S.A. 2011)

The male predominance is explained by the notion that the mean age at diagnosis is about a decade later in females than in males.[6;7] Indeed, other studies that included pediatric CVID patients also found a preponderance of males.[8-10] This could be due to higher numbers of class-switched and non-class-switched memory B cells in CVID females, and their higher baseline levels of serum IgA and IgM. Serum IgM levels have been linked to the number of X chromosomes carried by the individual; alternatively, the role of sex hormones in immune modulation could explain these dissimilarities.[11] The high prevalence of familial cases (25%) and TACI mutations may be related to the severity of these forms of CVID. TACI mutations predispose to CVID and may trigger the disease to manifest itself at an earlier age. Possibly, sporadic cases and patients without a TACI mutation present at a later age, and thus influence the constitution of the adult CVID population. Furthermore, there is the possibility of a selection bias; siblings of patients are watched vigorously and the threshold for immunological screening is low.

In conclusion, we found that children with CVID exhibit the same type of lymphocyte subset abnormalities as found in adult patients, but need to be compared with age-matched controls since cut-off levels for normality vary with age. Since we also noticed some differences with the adult CVID population, it is possible that pediatric CVID includes a subset of genetic defects that predispose to severer disease forms, which in turn lead to an earlier age of onset and perhaps a different disease course.

The continuum of B cell disorders; are cut-off values for CVID useful?

To establish an accurate and standardized diagnosis, a clear definition of CVID is required, although it will remain a heterogeneous patient group. The categorization of CVID patients into subgroups that are more homogeneous has provided some valuable insights on the prediction of CVID-related complications.[7;12;13] Classification may further enable etiologic research, as homogeneous patient groups enable studying immunopathogenetic mechanisms more effectively. Alternatively, it may be perilous to try and label different types of PID too strictly. An overtly stringent application of disease definitions may hinder etiologic and clinical research and perhaps even clinical care for the following reasons.

First, CVID even with a similar genetic background remains heterogeneous, with a very broad spectrum of immunological manifestations; examples include Ig serum titers that may range from near-normal to agammaglobulinemic, T cell abnormalities may be present or absent, and the fact that the quality of responses to vaccinations is diverse.

Second, there is a wide-ranging spectrum of clinical complications that may develop influenced by other host and environmental factors, and for example the rate, type and severity of infection differ per patient. As a result, establishing a strict disease definition including strict cut-off values is difficult and artificial, not only for CVID but also for other PIDs lacking a genetic diagnosis. Patients that resemble each other closely in the spectrum of the various diseases and even with a similar genetic background and within families may obtain a different diagnosis label and subsequently, their follow-up and eligibility for research studies may prove to be differential.

Third, in our studies, we have shown repeatedly that patients classified with 'CVID-like disease' are comparable to definite CVID at many fronts (chapter 2, 3, 5, 8, 12). These are all patients with characteristics similar to CVID patients, but that do not completely fulfill the diagnostic criteria established by the European Society for Immunodeficiencies.[14] However, CVID-like patients also have decreased class-switched memory B cells, severe infections, disease sequelae such as pulmonary abnormalities, intrinsic CVID-related complications, persistent gastrointestinal virus infections, and decreased calcium mobilization upon B cell receptor (BCR)-mediated activation. These findings confirm data published by Alachkar *et al.*, who demonstrated that percentages of $CD19^{+}CD27^{+}IgD^{-}$ B cells, but not serum Ig levels nor the diagnosis CVID versus specific antibody deficiency, correlated with complications such as bronchiectasis, splenomegaly and autoimmune phenomena.[15]

Fourth, particularly in pediatric CVID and CVID-like patients, disease characteristics may vary in time and re-evaluation of the original diagnosis may be necessary. The immunological defect may ameliorate, stabilize but also deteriorate from predominantly B cell dysfunction towards combined immunodeficiency (CID). Recently, a subset of patients with late onset CID (LOCID, all with clear manifestations of T cell malfunction) was described in patients previously diagnosed with CVID.[16] Likewise, in CVID patients with a diagnosis made at early age (<4-6 years), this diagnosis should be reconfirmed at a later stage. To this end, Ig levels and preferably specific antibody production should be re-evaluated, especially in the few patients that show signs of improved endogenous Ig production.

Lastly, there are many similarities between different PIDs, and pedigrees with different types of PID exist. This confirms that knowledge on human immunology is still limited; there are many interactions in immune pathways and protein functions still left to unravel; as a result, it is advisable to keep an open eye and wide approach in studying PID. At the same time, further insight in the origin of CVID and categorization may facilitate diagnosing specific disease subtypes, and thus enable timely initiation of adequate follow-up and treatment.

Etiopathogenetic research in B cell deficiencies; is it really worth it?

Most research efforts in the CVID field are geared towards discovering new genetic mutations, which are thus far described for less than 10% of the CVID patients. Noteworthy is that to date, surveillance and treatment regimens of these patients with known defects do not differ from patients of whom the molecular diagnosis has not been established. In other words, what is the use of uncovering more mutations?

We still have limited knowledge on the etiology of CVID, which precludes defining optimal guidelines for diagnosis, treatment and follow-up. As a result, clinical guidelines are currently based on the best available monitoring parameters, including numbers of class-switched memory B cells and signs of immune dysregulation. The ultimate goal is thus to relate the exact genetic defect to the functional immune defects and clinical CVID phenotype over time, providing prognostic information which is highly relevant for the patient and therapeutic interventions; CVID patients with TACI polymorphisms are for instance more vulnerable to the development of autoimmunity.[17;18] This approach will provide insight in the pathogenesis of disease and enable establishing adequate guidelines for clinical practice and follow-up. More insight in genetic and functional causes of PID also allows for perinatal screening for PID, or amongst family members. Furthermore, early identification of disease enables timely initiation of correct treatment, such as antibiotic prophylaxis, Ig replacement, or stem cell transplantation in cases of expected late onset CID. Eventually, enabling a genetic diagnosis may lead to corrective gene therapy or other tailor-made treatment strategies,[19] although application in the clinical setting still requires considerable time and effort. Taken together, etiopathogenetic research remains of major importance in CVID and other PIDs.

Nevertheless, it gradually becomes clear that predicting the clinical relevance of genetic mutations in PID-related genes remains exceedingly difficult, illustrated by the cases described in chapter 6. There are many reasons why genotype – phenotype relations can vary. For example, differences in penetrance may hinder the yield of genetic screening, as well as non-random X-chromosomal inactivation or somatic reverted mosaicism, which has been in many patients with Wiskott-Aldrich syndrome (WAS).[20] Modifying factors, either environmental or genetic, may affect the outcome of the genetic variant.[21]

To complicate matters further, the precise type and location of the mutation will influence the clinical outcome and should thus be determined.[22] This is exemplified by mutations in the *RAG* gene that are responsible for a spectrum of clinical phenotypes. Biallelic null mutations lead to T^-B^-SCID; Omenn syndrome patients have mono- or biallelic missense mutations; other missense mutations cause atypical SCID or Omenn syndrome; hypomorphic mutations cause leaky SCID and lastly, compound heterozygous mutations may lead to immunodeficiency with granulomas.[23;24] A second example of the complexity is that a mutation does not necessarily abolish or decrease the function of the gene-encoded protein, but may indeed lead to a gain of function. Both WAS and X-linked thrombocytopenia are caused by mutations in the WAS protein gene; WASp has an important role in cytoskeleton rearrangement, and many different genotypes and phenotypes have been described.[25;26] Mutations compromising normal auto-inhibition of WASp result in unregulated activation of the actin-related protein (Arp) 2/3 complex and increased actin polymerizing activity, which leads to a different primary immunodeficiency, i.e. X-linked neutropenia. These examples show the difficulties one may encounter when studying PID of monogenetic origin; since many cases of CVID are assumed to be polygenetic, these cases will be especially complicated to decipher. In order to unravel the etiology of a multigenetic

PID, genetic research should therefore be complemented by functional cellular and molecular studies.

B cell activation defects in CVID disorders; continuation of functional and genetic studies

In chapter 5, we hypothesized that patients with CVID disorders would have defective B cell activation and investigated early BCR signaling. We discovered that many CVID(-like) patients have decreased calcium mobilization upon triggering of the BCR signalosome, which was neither due to defective extracellular calcium influx through the plasma membrane calcium channels, nor to altered calcium release from the endoplasmic reticulum. Phosphorylation of tyrosine kinases pivotal in the BCR signaling cascade appeared to be normal. After excluding these pathways as the main cause of the defective calcium fluxes, we studied BCR signalosome events and noticed a defective interaction between the BCR and CD20. Consequently, we concluded that defective interaction of the BCR and its co-receptors may lead to ineffective B cell activation in CVID. The next challenge is to determine the genetic origin of this defect, which may be based in multiple genes involved in e.g. plasma membrane rearrangements such as lipid raft formation, cytoskeleton reorganization or the involved molecules themselves. Possibly, dissimilar genes are affected in different patients, leading to a common defective outcome.

In the continuation of these studies, a combination of Next Generation sequencing and functional assays would be an excellent approach.[27] Functional studies have limited the genes of interest to a specific area (chapter 4-5), and Next Generation sequencing is a rapid way to screen for mutations in these genes of interest. Further research in patients with a PID of unknown (polygenetic) etiology could therefore be performed as follows, on well-characterized PID patients *(figure 1).* Little patient material is needed to extract DNA, especially important in children. Once a certain genetic mutation has been found, *in silico* screening of online open access databases must be performed. This will provide information on the gene product's expression, function and conservancy amongst species; information on its primary and secondary structure may provide clues on the relevance of the mutation.

Second, *in vitro* cell culture-based studies should be executed. Depending on the available data and the cell type in which the gene is expressed, an artificial system to study the function of the mutated gene may be established. Fortunately, recent progress in immortalization, manipulation and culture of patient tissue enables experimental use for such studies. Antigen-specific B cells can be sorted and immortalized using Epstein-Barr virus and Toll-like receptor (TLR) ligation,[28;29] or by introduction B cell lymphoma (Bcl) 6 and Bcl-xL into B lymphocytes,[30] which generates stable B cell clones with an unaffected BCR signalosome.

To study other cell types, the induced pluripotent stem cells (iPSCs) may be an exciting option.[31] iPSCs may be regenerated from patients by genetic reprogramming of differentiated cells (e.g. fibroblasts) into pluripotent stem cells.[31] With the adequate stimuli, these iPSCs may be re-differentiated into virtually every cell type. Another elegant approach, increasingly resembling an *in vivo* system, would be the use of

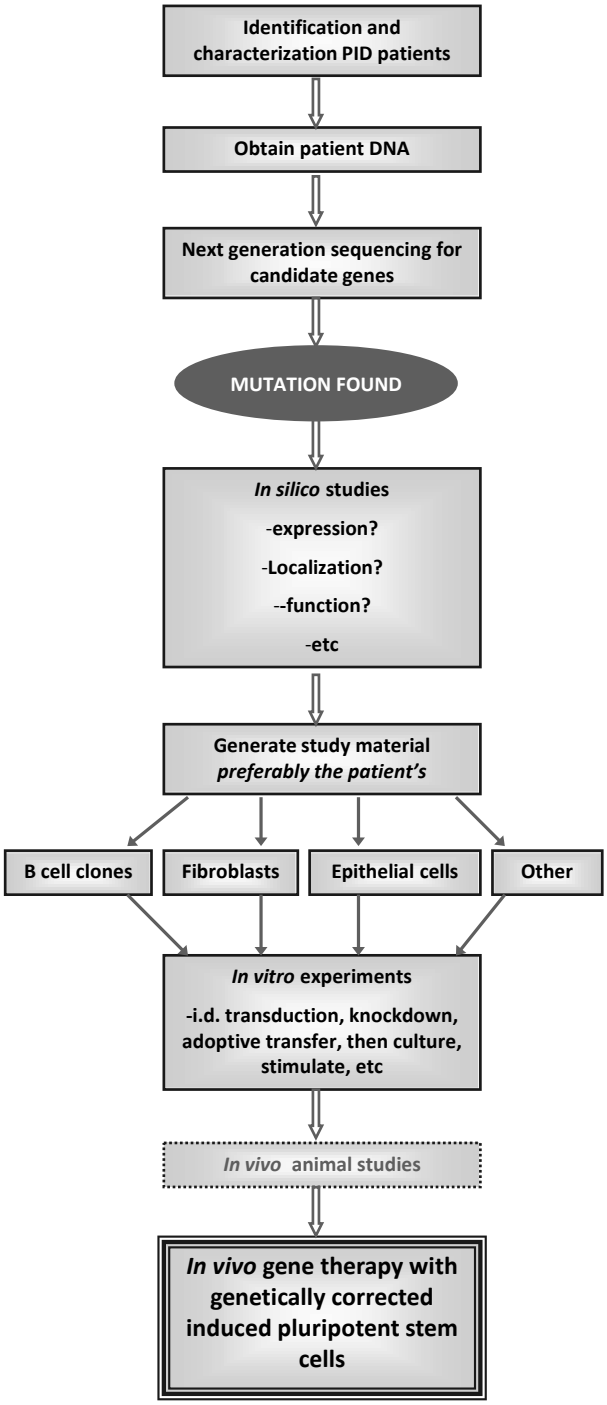

Figure 1. General strategy outline for future PID research.

organoids.[32] When culturing Lgr5$^+$ PSC on a matrix, these cells differentiate and form small organ-like structures, or organoids, resembling bowel or gastric tissue.[33] Generation of crypt-villus organoids from Lgr5$^+$ PSC isolated from gut biopsy specimens of CVID patients would enable studying mucosal immunity and epithelial function. Possibly, CVID patients which develop enteropathy have additional mucosal epithelium defects. This is supported by the description of defective TLR7 and TLR9 signaling in CVID;[34;35] as these TLRs are also expressed in intestinal epithelial cells, they may play a role in defective interaction between host and luminal flora.[36;37] The generation of crypt-villus organoids from CVID patients with enteropathy would enable the study of their epithelial cells. If eventually the addition of viruses or immune cells to these organoids could be realized, the possibilities to study host-microbe interactions will offer almost limitless opportunities that have lacked so far.

The availability of these human tissues will furthermore decrease the necessity of the use of *in vivo* studies using murine models. There is thus far no suitable animal model for CVID available, which will prove to remain complicated to elicit given the complexity and variability of the human disease. However, for more in-depth studies of already identified (monogenetic) defects, murine models could be supplementary. Finally, if true disease-causing mutations have been unraveled, attempts to develop a tailor-made genetic therapy should be made, by correcting mutations in iPSCs and returning them to the patient. In the first studies with oxidase-deficient neutrophils from X-linked chronic granulomatous disease, iPSCs show very promising results.[38]

Clinical studies in CVID; translation, collaboration, observation and documentation

Although fundamental studies are encouraging, one has to remain realistic regarding the expected time span until their outcomes will be clinically beneficial. The fundamental studies are expensive, time-consuming and difficult; application to patient material still requires optimalization. If a disease-causing genetic defect has been identified, there are still many hurdles in the development of corrective gene therapy. These studies are thus indispensable for the long-term PID research and care, but should not exclude clinical studies with perhaps more direct consequences.

The observational study described in chapter 12 shows that even relatively small clinical studies can have an impact on future research. Albeit a small study population with a longer follow-up period still to complete, some appealing observations have already been made. The presence of several persistent viruses in CVID disorders, but moreover their association with mucosal inflammation, strengthens our hypothesis of a relation between chronic viral infections and the development of CVID-related enteropathy. The persistence of viruses for over 3 months in some patients supports this notion (chapter 10-12). Accordingly, follow-up should be continued and the power of this study ought to be increased. It would be attractive to study other pediatric and adult CVID cohorts. The inclusion of patients with CVID as well as X-linked hypogammaglobulinemia (XLA),[39] a pure B cell defect[40] enables the comparison between T cell-dependent and -independent viral infections and mucosal alterations. In retrospect, the presence of enteric viruses in antibody deficient patients with

established enteropathy should be studied, e.g. by means of electron microscopy or polymerase chain reaction-based techniques. Finally, study of the microbiome in addition to the current panel of viruses would enable investigation of the interaction between mucosa, virus and microbiome.[41]

Similar conclusions can be drawn from our clinical study of pulmonary complications in pediatric CVID disorders. In chapter 7-9, we found that pulmonary anomalies are generally mild but not uncommon in pediatric CVID patients. Besides structural abnormalities such as airway wall thickening, children also display interstitial and parenchymal abnormalities, which probably result from a different immunopathogenesis (chapter 9). High-resolution computed tomography (HRCT) is the most sensitive and discriminative method to detect these complications. The diagnostic delay in our cohort is relatively short, high serum IgG trough levels are maintained and most children do not have pulmonary complaints. Therefore, detection of abnormalities in asymptomatic pediatric CVID patients on –supposedly- optimal treatment is a worrisome finding and urges confirmation in other cohorts. The development of a HRCT scoring method (chapter 8) greatly facilitates this inter-center evaluation by standardization radiological scoring. To further enhance standardization, automated quantitative measurements are exciting new software developments in radiology and may eventually be preferential over visual measurements.[42] After establishing these cross-sectional baseline findings, the real challenge lies in the patient treatment and follow-up. To date, there is no protocol available on how often pulmonary imaging should be repeated, and in which particular patients. The risk of silent progression of lung disease should be outweighed against the burden on health care and patient, especially regarding radiation exposure (appendix). To develop a high-quality evidence-based diagnostic protocol, meticulous documentation of clinical events and (antimicrobial) therapy during longitudinal observational studies is essential, as randomized clinical trials are preferably not performed in children. This way, the evolution of pulmonary disease can be studied in relation to treatment and disease parameters, enabling the detection of predictive factors and patients particularly vulnerable to lung damage. Evidently, it is advisable to increase power by extending the study population; the European chest CT study group provides an excellent platform. (www.chest-ct-group.eu) In addition, to address to potential benefit of low-dose steroids for asymptomatic patients with interstitial lung disease, a randomized multicenter clinical trial has been planned.

Finally, for every long-term study, adequate communication between care-takers is mandatory. Since CVID is a chronic disease continuing into adulthood, a communicative transition to internal medicine is crucial for continuation of follow-up, as well as close collaboration with pulmonologists, gastroenterologists, radiologists and other disciplines.

In this thesis we have described the immunological and pulmonary evaluation of pediatric CVID patients, investigated early B cell activation, and initiated a clinical study on gastrointestinal disease in antibody deficiency. These findings provide exciting ground-work for sustained, collaborative and translational studies in the future, for which we recommend the following.

FUTURE PROSPECTS

"A goal without a plan is just a wish."
Antoine de Saint-Exupery, writer, poet, and aviator (1900-1944)

General

Goal: to improve CVID research and eventually CVID care
Plan:

» Improve collaboration by implementation of universal protocols and accurate registration of data.
» Improve collaboration between different clinical specialties, and between medics and translational/basic laboratory researchers, with the aim to combine expertise and gather long-term follow-up data.

Etiologic studies

Goal: to identify new genetic disease-causing or disease-modifying genes in patients with CVID disorders, and to better understand the biological mechanism of the disease.
Plan:

» Perform Next Generation sequencing for preselected target genes on DNA of clinically and immunologically well-characterized CVID and other PID patients.
  - Detection of a potentially disease-causing genetic mutation should be followed by an extensive and accurate work-up, including functional laboratory studies.
  - Perform linkage studies and homozygosity mapping in specific families, followed by functional analyses.

Pulmonary studies

Goal: to define optimal individualized evidence-based diagnostic protocols to detect and monitor pulmonary complications in CVID and CVID-like disease, which will eventually dictate therapeutic regimens.
Plan:

» Establish large well-described patient cohorts; use universal scoring methods and protocolized follow-up to compare and combine data between centers.
» Continue studying other modalities in order to find the optimal pulmonary imaging protocol that can be most uniformly standardized and applied in all patient cohorts.
» Accurately register clinical events and treatment regimens in relation to the evolution of pulmonary abnormalities in order to evaluate the clinical benefit of protocolized follow-up.

Gastrointestinal studies

Goal: unravel the pathogenesis of enteropathy in antibody deficiencies, especially CVID, with the aim to prevent its development and improve its treatment.

Plan:

- Continue longitudinal observational study and increase its power, by accumulating follow-up years and augmenting the study population; i.e. other pediatric CVID cohorts, adult CVID patients, and other antibody deficiency patients
- Retrospectively study the presence of viruses in patients with established enteropathy
- Long-term: perform subsequent *in vitro* studies to study a causal relation between the viruses and enteropathy in CVID and related antibody deficiencies.
  - Study the microbiome for potential interactions with host and viruses
  - Study interaction of virus and immune system of the patient by developing and applying experimental assays
  - Study epithelial cells of CVID patients with enteropathy using organoids
  - Study the interaction between viruses (and microbiome) and epithelial cells using organoid-type culture systems

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SUMMARY & SAMENVATTING

SUMMARY

Primary immunodeficiency patients suffer from monogenetic or polygenetic defects in the immune system that render them susceptible to a variety of infections. Common variable immunodeficiency (CVID) is the most common primary immunodeficiency requiring treatment. Despite significant progress in fundamental research and treatment strategies, many issues in the origins of CVID still need to be addressed to provide tailored health care. For example, the development of classification systems has enabled distinction of patients prone to develop disease-related complications such as autoimmune cytopenia and granulomatous disease, but the origination of these presumed causal relations remains to be established. Further, disease-causing monogenetic defects in e.g. B cell co-receptors were found, which have provided important clues for understanding human immunology, but a molecular diagnosis remains limited to a neglible fraction of patients. And finally, treatment has improved significantly in the recent decades, but nevertheless, many patients suffer on a daily basis from the consequences of their disease.

Phenotypic and functional B cell abnormalities in CVID

This thesis provides various insights on the pathogenesis and complications of CVID, which were obtained by studies in children with CVID and related antibody deficiencies. As current literature is mainly based on research in adult CVID patients, it had thus far been uncertain whether pediatric CVID is the same disease displayed a younger population, or a dissimilar disease entity. In **chapter 2-3**, we show that pediatric CVID disorders are analogous to adult CVID regarding lymphocyte characteristics, with increased numbers of transitional B cells accompanied by low memory populations, in comparison to age-related reference values. As a result, CVID classifications show a different patient distribution when applied to children and are thus inapt for this population. Akin to adults, CVID-related complications are associated with severely decreased percentages of class-switched memory B cells; their incidence is however lower in children.

The etiology of CVID remains to be elucidated, but probably originates in the B lymphocyte for the majority of patients **(chapter 4)**. We have investigated early B cell activation upon triggering of the B cell receptor (BCR). In **chapter 5**, we demonstrate that BCR-mediated calcium signaling is disturbed in a significant proportion of pediatric CVID(-like) patients. The defect correlates with disturbed plasmablast differentiation *in vitro* and appears to occur upstream in the BCR signaling cascade, i.e. endoplasmic reticulum Ca^{2+} storage and depletion were normal, as well as calcium influx via plasma membrane channels. These data suggest that defective plasma membrane lateral interaction of the BCR with its co-molecules is a pivotal pathogenetic mechanism in CVID, and is supported by our finding of defective B cell surface dissociation of CD20 and the BCR. The complexity of studying heterogeneous multifactorial disorders such as CVID is further demonstrated in **chapter 6**, describing three siblings with identical mutations in the TACI susceptibility gene, but with clinical phenotypes ranging from completely asymptomatic to severe CVID with refractory autoimmune symptoms.

Pulmonary complications in pediatric CVID disorders

Pulmonary abnormalities are the most frequent complications in CVID, causing significant morbidity and at times mortality. Consequently, accurate detection is of major importance, and preferably performed early in the course of disease development when adequate treatment options are still available. Scientific literature unambiguously shows that high-resolution computed tomography (HRCT) is the most sensitive non-invasive method to detect pulmonary anomalies in CVID **(chapter 7)**. Though most research was performed in adult CVID patients, a few studies show that structural abnormalities such as bronchiectasis can already occur in childhood CVID. In **chapter 8**, we describe for the first time in pediatric CVID patients comparative data on interstitial or parenchymal lung disease. While pulmonary abnormalities in pediatric CVID are common but generally mild as rated by HRCT, they may occur despite what we consider optimal treatment regimens and despite the lack of pulmonary symptoms. In this chapter, we additionally report the first HRCT scoring method that is specifically designed for CVID and related antibody deficiencies. In **chapter 9**, we report the validation of the HRCT scoring system**,** which revealed that this diagnostic method has sensitivity and discriminative potential superior to conventional chest X-ray and pulmonary function tests. This chapter furthermore confirms that CVID-related lung abnormalities develop via different pathogenetic mechanisms. Accordingly, the separation of complications into two categories (structural airway disease and interstitial or parenchymal lung disease) revealed that the patients concerned display dissimilar clinical and immunological characteristics. Pneumonias occurred more often and disease duration was relatively longer in patients with structural airway disease, suggesting that structural lung complications probably result from structural damage due to cumulative infectious events. Concerning pathogenesis of interstitial lung disease, pathology appears to result from a broader immune-related dysregulation: these patients more often suffer from autoimmune and lymphoproliferative disorders, and display several lymphocyte alterations; most notably a severe decrease of memory B cells and a trend towards increased $CD8^+$ cytotoxic memory and effector T cells.

Gastrointestinal manifestations in CVID and related antibody disorders

Enteropathy is the most lethal non-malignant intrinsic complication in CVID, with typically an unsatisfactory response to treatment. The case of CVID enteropathy described in **chapter 10** demonstrates the exceeding challenges in reaching complete disease remission: a combinatory regimen of steroids and tacrolimus only accomplished partial remission. Second, it illustrates that human parechovirus is nearly impossible to eradicate in the agammaglobulinemic patients, and may persist in the gastrointestinal tract for many years. Review of the literature confirmed that persistent viral shedding occurs occasionally in the immunocompromised individual, and very sporadically in immunocompetent subjects **(chapter 11)**. Several cases of chronic enteroviral infections in humoral immunodeficiency have been reported; cohort studies however show that this does not occur on a regular basis. Whether there is a relation between persistent enteric virus infections and enteropathy in

immunodeficiency remains to be elucidated. To this end, a longitudinal study has been initiated and promising preliminary data are described in **chapter 12**. Children with CVID and related antibody deficiencies suffered significantly more often from gastrointestinal discomfort than healthy children. These symptoms were not merely functional, but associated with intestinal inflammation. In addition, common enteric infections were numerous and associated with intestinal inflammation in pediatric CVID, but not in healthy children.

This thesis involved the phenotypic and mechanistic clarification of CVID disease and its complications, which should eventually facilitate molecular diagnoses, prevention of complications and optimalization of therapeutic options. Future studies to consecutively address these issues are suggested in the discussion.

NEDERLANDSE SAMENVATTING

Patiënten met een primaire immuundeficiëntie hebben door een defect in één of meerdere genen een stoornis van het afweersysteem. Hierdoor zijn zij extra vatbaar voor infecties. *Common variable immunodeficiency* (CVID) is de meest voorkomende primaire immuundeficiëntie waarvoor behandeling noodzakelijk is. Hoewel er sterke vooruitgang is geboekt in begrip en behandeling van deze ziekte, zijn nog vele facetten in het ontstaan van CVID onduidelijk. Een helder inzicht in de pathogenese van CVID is van belang om adequate en patiëntgerichte therapie te kunnen bieden. De ontwikkeling van CVID classificatie systemen heeft er bijvoorbeeld toe geleid dat patiënten met een verwacht groot risico op ziektegerelateerde complicaties zoals auto-immuun cytopenieën en granulomateuze aandoeningen sneller geïdentificeerd kunnen worden, al blijft de oorzaak van deze complicaties onbekend. Verder zijn ziekteveroorzakende erfelijke defecten gevonden, bijvoorbeeld afwijkingen in genen die coderen voor B cel coreceptoren. Dit type bevindingen heeft een belangrijke bijdrage geleverd aan de kennis van het humane immuunsysteem. Bij het merendeel van de CVID patiënten is de moleculaire diagnose nog niet bekend. Ook blijft voor veel CVID patiënten de morbiditeit hoog, ondanks een sterk verbeterde behandeling in de afgelopen decennia.

Fenotypische en functionele B cel afwijkingen in CVID

Dit proefschrift beschrijft nieuwe inzichten in de pathogenese van CVID en complicaties van de ziekte bij jonge kinderen. De studies zijn verricht bij kinderen met CVID en met antistofdeficiëntie. Aangezien de literatuur over CVID vooral is gebaseerd op onderzoek in volwassen CVID patiënten, was het tot nog toe onduidelijk of pediatrische CVID dezelfde ziekte is, die zich manifesteert in een jongere populatie, of een ander ziektebeeld met een andere oorzaak en beloop. In de **hoofdstukken 2-3** laten wij zien dat de pediatrische CVID aandoeningen vergelijkbaar lijken met die in volwassen CVID patiënten als men kijkt naar kenmerken van afweercellen als B lymfocyten; transitionele B cel aantallen zijn verhoogd, terwijl populaties B geheugencellen verlaagd zijn, ten opzichte van leeftijdsafhankelijke referentiewaarden. De huidige CVID classificaties, die gebaseerd zijn op volwassen patiënten, laten een andere patiëntverdeling zien wanneer zij toegepast worden op kinderen en zijn dus ongeschikt voor applicatie bij kinderen. Net als bij volwassenen met CVID zijn CVID-gerelateerde complicaties in kinderen geassocieerd met sterk verlaagde percentages van immunoglobuline *class-switched* B geheugencellen, maar de incidentie van deze complicaties is in kinderen lager.

De etiologie van CVID is nog grotendeels onopgehelderd. Het kan voor veel CVID patiënten variëren, maar het is aannemelijk dat de oorzaak bij de B cel ligt voor een omvangrijk aantal patiënten **(hoofdstuk 4)**. Wij hebben vroege B cel activatie in kinderen met CVID bestudeerd door middel van B celreceptor (BCR) stimulatie. In **hoofdstuk 5** laten we zien dat BCR-gemedieerde calciumsignalering is verstoord in een groep van kinderen met CVID. Het defect correleert met verstoorde plasmablast differentiatie *in vitro* en zit vermoedelijk in het initiële deel van de BCR

signaleringscascade, omdat de calciumopslag en -afgifte van het endoplasmatisch reticulum normaal zijn, evenals de calciuminstroom door plasmamembraan kanalen. Onze data suggereren dat een defecte interactie tussen de BCR en geassocieerde comoleculen in het plasmamembraan een relevant pathogenetisch mechanisme in CVID is. Dit wordt ondersteund door onze bevinding dat dissociatie van de BCR van CD20 op het B celoppervlak is verstoord.

De complexiteit van onderzoek naar heterogene multifactoriële ziektebeelden zoals CVID wordt benadrukt in **hoofdstuk 6**, waarin drie familieleden met identieke varianten in het *TACI* gen worden beschreven. Bepaalde varianten in TACI predisponeren tot het ontwikkelen van CVID; maar ondanks dat de familieleden een identiek genotype hadden, varieerden de klinische fenotypes bij hen van compleet gezond tot ernstige CVID met onbehandelbare auto-immuun symptomen.

Longcomplicaties in pediatrische CVID aandoeningen

Pulmonale afwijkingen zijn de meest voorkomende complicaties van CVID en leiden tot aanzienlijke morbiditeit en soms mortaliteit. Nauwkeurige detectie in een vroeg stadium van de ziekte is van groot belang, zodat tijdige behandeling tot de mogelijkheden behoort. Wetenschappelijke literatuur laat zien dat *high resolution computed tomography* (HRCT) de meest sensitieve en niet-invasieve detectiemethode is om longafwijkingen in CVID op te sporen **(hoofdstuk 7)**. Hoewel het meeste onderzoek naar afwijkingen in de longen is verricht op volwassen CVID patiënten, laat een aantal studies zien dat structurele luchtwegafwijkingen zoals bronchiectasieën ook al op de kinderleeftijd kunnen optreden. In **hoofdstuk 8** beschrijven wij de eerste vergelijkende data over interstitiële en parenchymateuze longafwijkingen in kinderen met CVID aandoeningen. Hoewel longafwijkingen al frequent aanwezig zijn in kinderen met CVID op HRCT beelden, zijn ze doorgaans mild van aard. Deze afwijkingen treden echter op ondanks veronderstelde optimale behandelmethoden met gammaglobulinen en uiten zich niet altijd klinisch. In dit hoofdstuk beschrijven we tevens de eerste HRCT scoringsmethode die specifiek is ontwikkeld voor CVID en gerelateerde antistofdeficiënties in ons onderzoek. Aansluitend wordt deze scoringsmethode in **hoofdstuk 9** gevalideerd. Dit laat zien dat HRCT sensitiever is en beter onderscheid kan maken tussen de diverse longafwijkingen dan conventionele röntgenfoto's of longfunctietesten. Tevens wordt in dit hoofdstuk duidelijk dat CVID-gerelateerde longafwijkingen voortvloeien uit verschillende pathogenetische mechanismen. De verdeling van complicaties in twee categorieën, te weten structurele luchtwegveranderingen enerzijds en interstitiële/parenchymateuze longafwijkingen anderzijds, laat zien dat de gecategoriseerde patiënten ook verschillende klinische en immunologische kenmerken vertonen. Patiënten met structurele luchtwegafwijkingen hebben vaker een longontsteking doorgemaakt en zijn relatief langer bekend met de diagnose CVID dan patiënten zonder pulmonale afwijkingen. Dit suggereert dat hun longafwijkingen het gevolg zijn van cumulatieve luchtweginfecties. Interstitiële en parenchymateuze longziekten lijken te resulteren uit een bredere verstoring van de immuunregulatie, omdat deze patiënten ook vaker lijden aan auto-immuun en lymfoproliferatieve complicaties. Bovendien hebben ze diverse lymfocytaire

afwijkingen; meest opvallend zijn een sterke afname van B geheugencellen en een trend in toename van cytotoxische $CD8^+$ T effector- en geheugencellen.

Gastrointestinale manifestaties van CVID en gerelateerde antistofdeficiënties

Enteropathie heeft van de niet-kwaadaardige ziektegerelateerde complicaties van CVID de hoogste mortaliteit en is doorgaans moeilijk te behandelen. Een casus van een CVID patiënt met enteropathie is beschreven in **hoofdstuk 10** en illustreert dat complete ziekteremissie niet eenvoudig te verkrijgen is; combinatietherapie van corticosteroïden en tacrolimus leidde slechts tot gedeeltelijke remissie. Daarnaast demonstreert dit hoofdstuk hoe moeizaam eradicatie van humaan parechovirus in patiënten met agammaglobulinemie verloopt en dat het virus jarenlang in het maagdarmstelsel kan persisteren. Een literatuurstudie bevestigt dat persisterende virusuitscheiding nu en dan optreedt in immuungecompromitteerde patiënten en sporadisch in personen met een normaal immuunsysteem **(hoofdstuk 11)**. Hoewel er diverse casus van chronische enterale virusinfecties in humorale immuundeficiëntie zijn beschreven, is het tot op heden onbekend of er een verband bestaat tussen persisterende enterale virusinfecties en de ontwikkeling van enteropathie in immuundeficiëntie. Wij hebben daarom een longitudinale studie gestart, waarvan enkele veelbelovende resultaten uiteengezet worden in **hoofdstuk 12**. Kinderen met CVID en gerelateerde antistofdeficiënties hebben significant vaker gastrointestinale symptomen dan gezonde kinderen. Dit betreffen niet louter functionele buikklachten, aangezien zij gepaard gaan met biochemische kenmerken van slijmvliesontsteking. Daarnaast hebben wij diverse veel voorkomende gastrointestinale virussen gevonden; deze virusinfecties zijn geassocieerd met slijmvliesontsteking in de CVID patiënten, maar niet in gezonde controlekinderen.

Samenvattend beschrijft dit proefschrift enkele fenotypische en mechanistische ontrafelingen van CVID en bijbehorende complicaties, welke uiteindelijk zullen bijdragen aan het stellen van een moleculaire diagnose, aan de preventie van complicaties en aan de optimalisatie van behandelingsstrategieën.

APPENDIX
HIGH-RESOLUTION COMPUTED TOMOGRAPHY IN PEDIATRIC COMMON VARIABLE IMMUNODEFICIENCY: RISKS AND BENEFITS

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Suzanne W.J. Terheggen-Lagro, and Joris M. van Montfrans

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Editor,

We recently reviewed the literature on pulmonary complications in children with common variable immunodeficiency (CVID) and concluded that pulmonary complications are common, that they may be severe, and that high-resolution computed tomography (HRCT) is the most sensitive method to detect pulmonary complications in these patients.[1] Recent findings in our pediatric CVID cohort confirm that HRCT abnormalities are common[2] and can be identified even in children with normal chest X rays or spirometry (Van de Ven *et al*. submitted). In response, Turner *et al*. stated their concerns regarding radiation exposure with chest HRCT in children with CVID. We agree that one should be careful with roentgen techniques, particularly in patients with CVID, as they are more prone to develop hematological malignancies and more sensitive to radiation.[3] Roentgen tests must be obtained at a dose as low as reasonably achievable and when this is adhered to, we believe that risks are generally small and the decision to scan mainly concerns the patient benefit side of the risk-benefit balance.

First, for HRCT to be beneficial, it should demonstrate CVID-related pulmonary complications reliably and reproducibly. Second, it must be accurate and show additional value to easier and safer tests, and third it should lead to changes in management and improvement in quality-adjusted life-years at reasonable costs.

First, we showed that even at low-dose HRCT can demonstrate CVID-related pulmonary complications in children by using a simple end-inspiratory and end-expiratory breathhold technique. The CVID-related abnormalities could be evaluated reproducibly by observers with moderate levels of chest CT imaging experience.

Second, pulmonary complications contribute significantly to the morbidity (and in some cases mortality) of a large part of the pediatric and of the majority of the adult CVID population and we believe that evidence of the extra diagnostic value of HRCT is accumulating.[1;4-7] Our experiences differ from those by Turner *et al*. who question whether the supplementary value of HRCT outweighs the risk of radiation, as complications such as bronchiectasis can easily be diagnosed using alternative techniques. For example, persistent sputum production with positive microscopy was reported to correlate well with HRCT evidence of bronchiectasis in CVID patients. However, in the referred retrospective study, sputum was only available from 25 of the 47 patients (53%) and the incidence of bronchiectasis was not described in the patient population without productive cough.[8] The majority of studies on pulmonary complications in CVID is retrospective and relates to selected CVID patients undergoing HRCT for clinical purposes, thus carrying a high risk of selection bias. Also, Turner *et al.* do not mention parenchymal and interstitial lung disease (ILD),[9;10] which are common in CVID, even in childhood.[2] It is important to distinguish interstitial lung disease from airway disease, as interstitial lung disease is associated with decreased survival[11] and requires a different, immunosuppressive treatment regimen.[12] In conclusion, HRCT is indispensable for accurate detection of pulmonary complications in (pediatric) CVID patients.

Third, for HRCT to be able to improve outcome, effective treatment is needed. Turner *et al.* are correct in pointing out that optimal treatment has not been defined. The relatively high incidence of chronic lung disease in adult CVID however underlines that improved prevention and treatment of pulmonary complications is urgently needed. To prevent progression of structural lung changes, literature suggests that prophylactic antibiotics and high trough levels of immunoglobulin are beneficial,[9;13] although robust randomized-controlled trials defining optimal therapy are not available. ILD requires different therapy, and usually responds well to immunosuppression. In our cohort, nodules on HRCT disappeared after steroid treatment, suggesting that ILD is still reversible at an early stage. It remains unclear whether treatment of asymptomatic lesions is advisable to prevent further progression. Additionally, ILD appears to be initially asymptomatic, which implies that screening of patients at risk (shown by the presence of other autoimmune phenomena and severely affected lymphocyte subsets; own unpublished observations) could facilitate early detection and henceforth prevent progression of CVID-related ILD.

In conclusion, HRCT performed by using low-dose HRCT can vividly and reproducibly demonstrate CVID-related airway and interstitial complications, even in asymptomatic patients with normal chest radiograph and lung function. To develop evidence-based diagnostic and therapeutic protocols for CVID, much work remains to be done. We agree that at present there is insufficient evidence to recommend a yearly scanning protocol for all (asymptomatic) pediatric CVID patients. Given the low prevalence of CVID, multi-center studies e.g. as currently initiated by the Chest CT in Antibody Deficiency Group[5;14] will be needed. Current management of pulmonary complications in CVID requires a multitude of clinical skills and expertise, and the indication for HRCT scanning is currently included in this expert judgment for an individual patient. Collaborative studies that include HRCT will lead to more structural and evidence-based diagnosis and treatment of CVID-related pulmonary complications.

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"It is good to have an end to journey toward;
but it is the journey that matters, in the end"
Ernest Hemingway, writer, (1899-1961)

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* Hoewel dit in de praktijk het meest voorkomende scenario is, heb ik het beschrijven hiervan wijselijk overgeslagen om het boekje niet al te dik te maken

CURRICULUM VITAE

Annick van de Ven was born on March 13th, 1982 in Nuenen, the Netherlands. In 2000 she completed secondary school *(cum laude)* at the Strabrecht College in Geldrop, the Netherlands. Subsequently, she studied Spanish language, culture and literature in Salamanca and Granada, Spain, and obtained the 'Diploma Superior de Español como lengua extranjera' (D.E.L.E. Superior). In 2001 she started medical training at Utrecht University, the Netherlands. During this training she performed an elective clinical rotation in General Surgery at the Hospital Civil Fray Antonio Alcalde, Guadalajara, Mexico, and an Obstetrics and Gynaecology rotation at the Stellenbosch University and Tygerberg Hospital in Cape Town, South Africa. Scientific rotations were performed at the Pediatric Immunology laboratory of prof. dr. B.J. Prakken at the Wilhelmina Children's Hospital and University Medical Center Utrecht, and at the department of Pediatrics at University of California, San Diego (UCSD). At UCSD, she worked for 9 months under supervision of prof. S. Albani at induction of regulatory T cells by means of epitope-specific immune therapy in rheumatoid arthritis. In 2007, she passed the Master's (*cum laude*) and medical finals and started her PhD studies in January 2008 at the department of Pediatric Immunology and Infectious Diseases, Wilhelmina Children's Hospital and University Medical Center Utrecht (prof. dr. E.A.M. Sanders). Results of this research are presented in this thesis.

LIST OF PUBLICATIONS

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