NON-INFECTIOUS COMPLICATIONS OF PRIMARY ANTIBODY DEFICIENCY

EDITED BY: Giuseppe Spadaro, Isabella Quinti, Stephen Jolles and Antonio Condino-Neto

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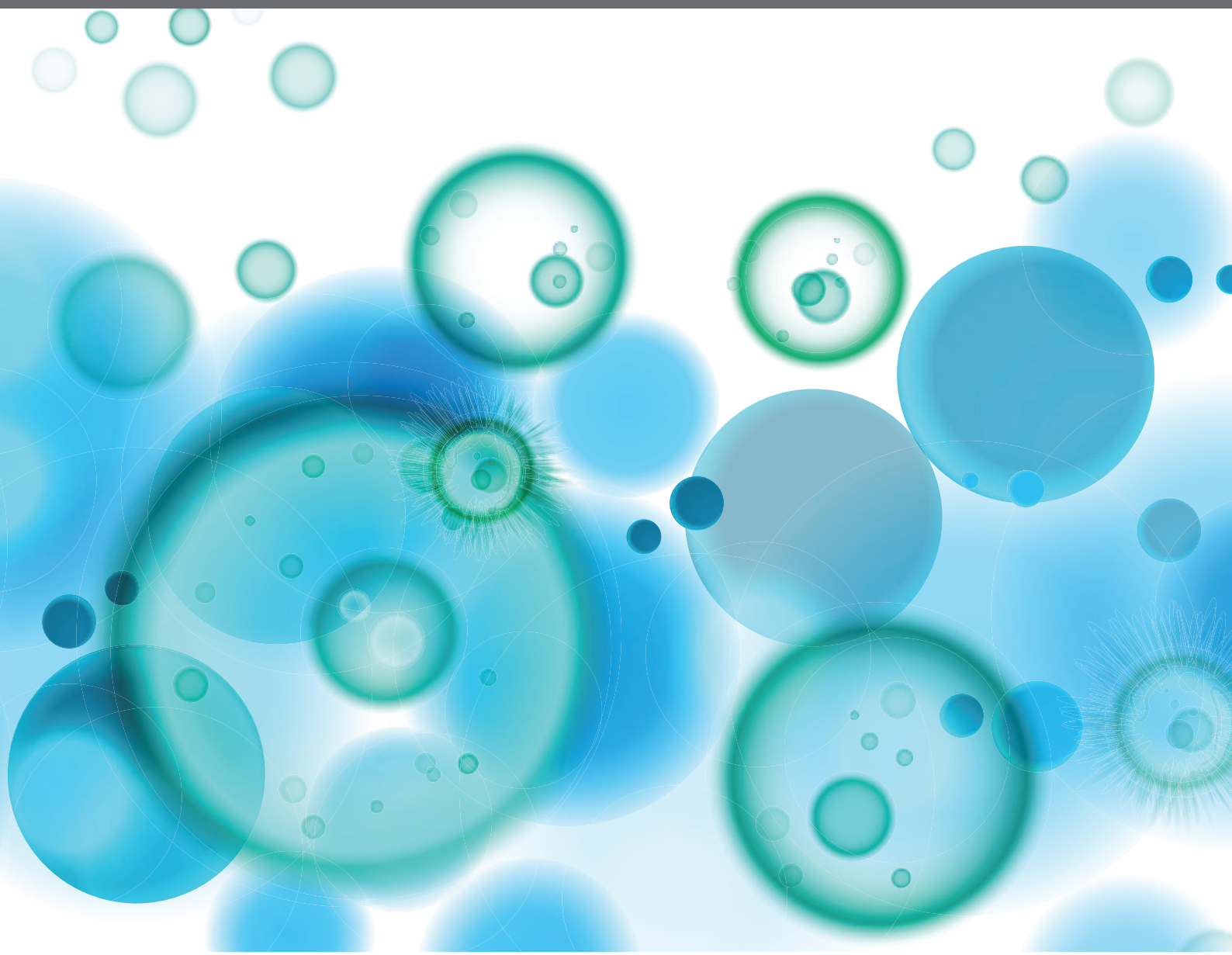

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NON-INFECTIOUS COMPLICATIONS OF PRIMARY ANTIBODY DEFICIENCY

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EDITORIAL
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Editorial: The Complexity of Primary Antibody Deficiencies

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Keywords: primary antibody deficiencies agammaglobulinemia, common variable immune deficiencies, chronic lung disease, liver diseases, endocrine diseases, T helper follicular cells, free light chains

Editorial on the Research Topic

Non-Infectious Complications of Primary Antibody Deficiency

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Primary antibody deficiencies represent by far the largest group of primary immunodeficiencies (PID) at 56% (ESID Registry), however the proportion of patients in whom antibody deficiency represents a component of their condition is greater still at around 75% (1). It has become clear over recent years that while the vast majority of such patients experience recurrent infections a significant proportion are also affected by non-infectious dysregulatory complications which include malignancy, autoimmunity, inflammation and allergy (**Figure 1**). The increasingly complex manifestations of Primary Antibody Deficiencies have a major impact on the clinical management of patients and raise diagnostic and therapeutic challenges (2). This Research Topic draws together a series of reports focusing on a range of these non-infectious complications and their clinical implications.

GENETICS

In recent years, our understanding of genetics of primary antibody deficiencies (PAD) (1) further progressed unraveling an independent role of minor and major histocompatibility complex genes and common genetic variants as well as monogenic forms as shown by Abolhassani et al. who identified the most significant partial haplotype linked with the unsolved CVID as W*01:01:01-DMA*01:01:01-DMB*01:03:01:02-TAP1*01:01:01. Beside the high number of BTK mutations so far described (2), some atypical clinical manifestations have been described in XLA patients and linked to a novel hemizygous c.1632-1G>A mutation in the *BTK* gene as shown by Han et al. in a child with atypical X-Linked Agammaglobulinemia and recurrent hemophagocytosis (HLH) whose remission of HLH episodes was finally achieved after he received monthly Ig replacement therapy as the only treatment for HLH.

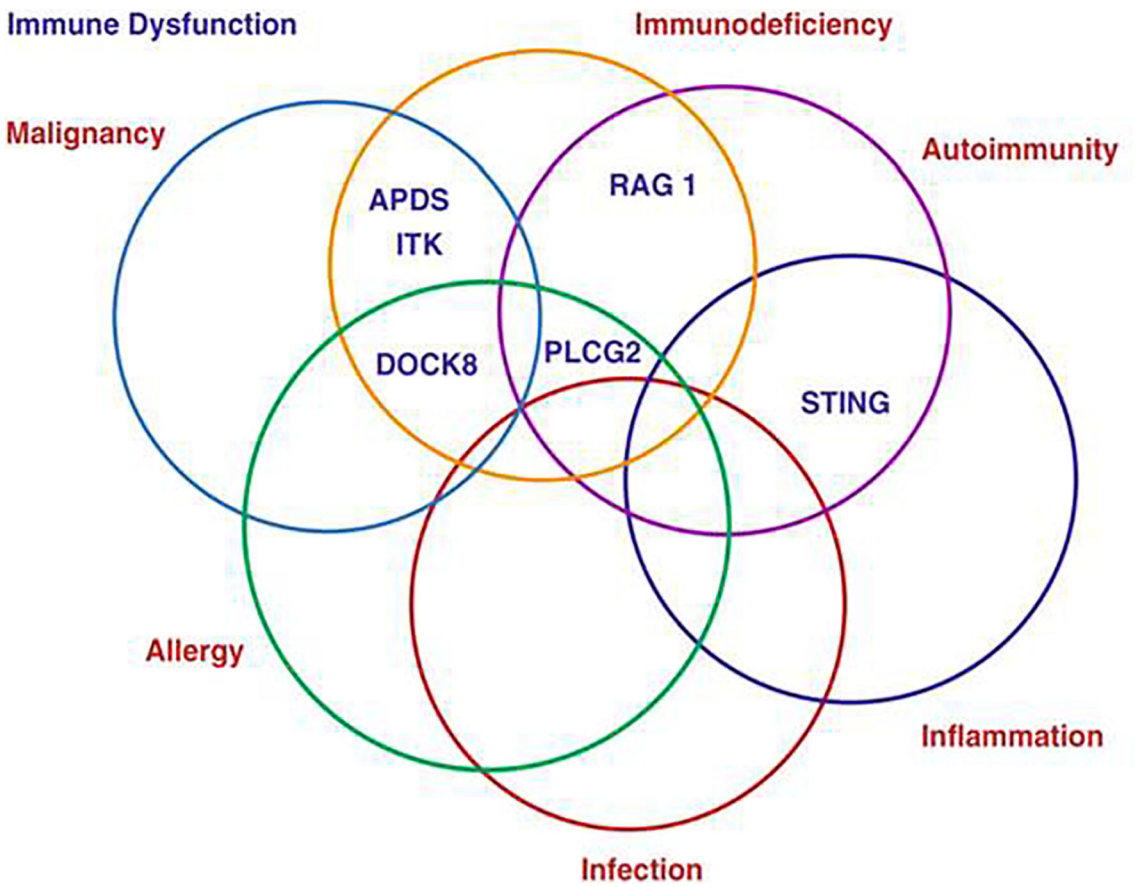

FIGURE 1 | The complexity of Primary Antibody Deficiencies.

PATIENT MANAGEMENT

As outlined, further advances in understanding key aspects of primary antibody deficiency associated conditions should assist in advances in diagnosis and management. New aspects of the clinical complexity of PAD was reported by Coopmans et al. who suggested that an assessment of the endocrine axis should be considered since a high prevalence of both anterior pituitary and end-organ endocrine dysfunction (secondary hypothyroidism, secondary hypogonadism, premature ovarian failure, primary testicular failure, partial adrenal insufficiency, severe growth or mild hormone deficiency) were identified in adult PAD patients causing a considerable health burden. A detailed description of CVID-associated non-infectious complication was reported by Ho and Cunningham-Rundles. Autoimmunity, chronic lung disease, lymphoid hyperplasia/splenomegaly, liver disease, granulomas, gastrointestinal disease, lymphoma and other malignancies were found in two third of CVID patients. These complications may be present in the same patient, and progress to a wide spectrum of associated sequelae. An aggressive multidisciplinary approach may reduce such progression. Further exacerbations, as for permanent lung damage and bronchiectasis, may develop even in the absence of known infections, as shown by Wall et al. Similarly, in the liver, granulomas and nodular regenerative hyperplasia progressing to portal hypertension (3) affected about 50% of CVID patients without or with monogenic forms included in the clinical spectrum of CVID such as ICOS, NFKB1, NFKB2, CTLA-4, PI3Kδ pathway, ADA2, and IL21-R genetic defects, as shown by Antonio Pecoraro et al.

PATHOGENESIS

The hallmarks of CVID are hypogammaglobulinemia, low frequency of isotype-switched memory B cells, and compromised B-cell differentiation into memory or antibody-secreting cells (4). Further insight on cellular defects underlying CVID pathogenesis are illustrated by Carsetti et al. who showed that circulating IgM memory B cells have a distinctive role in mucosal protection and suggested the existence of a functional gut-spleen axis where TACI-expressing IgM memory B cells producing IgA were localized under the epithelial cell layer where the TACI ligand APRIL was extremely abundant. The impairment of mucosal immunity might result in less diverse and significantly altered bacterial, but not fungal gut microbiota, in CVID patients, apparently associated with a more severe disease phenotype as shown by Fiedorová et al. Although described as a B cell intrinsic disease, numerous abnormalities have been reported in other immune cell compartments as in follicular

helper T cells, a CD4+ T cell population specialized in B cell help as described by Le Saos-Patrinos et al., and by Gereige and Maglione who address the aspects of immune dysregulation associated with autoimmunity, including elevations of T helper type 1 and follicular helper T cells and B cells expressing low levels of CD21 as well as a decrease in regulatory T cells.

DIAGNOSIS

An often unresolved aspect in the management of antibody deficiencies is the differential diagnosis with secondary forms of hypogammaglobulimias, and in particular those associated with lymphoproliferative diseases (5). Scarpa at al. proposed that serum free light chains analysis might have a role in differential diagnosis of CVID from other causes of hypogammaglobulinemia and in the early detection of monoclonal lymphoproliferation occurring over years. Overall, CVID patients presented a low κ and λ chain concentration. The most common pattern was κ–λ–, followed by κ–λ+, κ+λ+, and κ+λ–, while in secondary forms it was κ+λ+.

CONTROVERSIES

Experience of solid organ transplantations, and hematopoietic stem cell transplantation in patients with primary antibody deficiency remains limited and this aspect of management needs further study alongside the developing potential of gene therapy and gene editing.

CONCLUSIONS

Together the articles comprising this Research Topic provide important and timely updates about the current status of non-infectious diseases in primary antibody deficiencies, and in particular in CVID. Each report raises questions and indicates aspects that require further attention and scientific enquiry.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Bacterial but Not Fungal Gut Microbiota Alterations Are Associated With Common Variable Immunodeficiency (CVID) Phenotype

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Common Variable Immunodeficiency (CVID) is the most frequent symptomatic immune disorder characterized by reduced serum immunoglobulins. Patients often suffer from infectious and serious non-infectious complications which impact their life tremendously. The monogenic cause has been revealed in a minority of patients so far, indicating the role of multiple genes and environmental factors in CVID etiology. Using 16S and ITS rRNA amplicon sequencing, we analyzed the bacterial and fungal gut microbiota, respectively, in a group of 55 participants constituting of CVID patients and matched healthy controls including 16 case-control pairs living in the same household, to explore possible associations between gut microbiota composition and disease phenotype. We revealed less diverse and significantly altered bacterial but not fungal gut microbiota in CVID patients, which additionally appeared to be associated with a more severe disease phenotype. The factor of sharing the same household impacted both bacterial and fungal microbiome data significantly, although not as strongly as CVID diagnosis in bacterial assessment. Overall, our results suggest that gut bacterial microbiota is altered in CVID patients and may be one of the missing environmental drivers contributing to some of the symptoms and disease severity. Paired samples serving as controls will provide a better resolution between disease-related dysbiosis and other environmental confounders in future studies.

Keywords: CVID, IgA, gut microbiota, gut microbiome, gut mycobiota, gut mycobiome, fungal microbiota, fungal microbiome

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INTRODUCTION

Common Variable Immunodeficiency (CVID) is the most frequent symptomatic immune disorder, estimated to affect 1 in 25,000 people worldwide, although the prevalence can vary across different countries (1, 2). CVID includes clinically and genetically heterogeneous disorders characterized by reduced serum immunoglobulins IgG, IgA, and inconstantly also IgM. As a result of a defect in antibody production, most patients suffer from severe, recurrent infections, mainly of the respiratory and gastrointestinal tract, and have impaired vaccine responses (3, 4). These often manifest as autoimmune complications or inflammatory conditions. Malignancy occurs more frequently in CVID than in the general population (5). Owing to clinical heterogeneity, the disease cannot be determined by a single clinical or laboratory feature. Various diagnostic criteria have been proposed for CVID diagnosis (1, 3, 6, 7). The monogenic cause was revealed in <10% of patients so far, indicating both genetic and environmental factors' contribution in CVID etiology (3, 8).

Recently, human gut microbiota research and its implications in health and disease has attracted tremendous attention. Technological progress in high throughput sequencing has enabled us to associate alterations in gut microbiota composition to a wide variety of human diseases (9, 10) and the expanding knowledge of how gut microbiota affects the host led to new clinical procedure development (11). Recent murine and human studies found gut microbiota to be a crucial factor in shaping and modulating immune system responses (12–14). Gut microbiota modulates the host's immune system via its structural components and metabolites (15, 16). Microbiota-derived metabolites maintain a homeostatic environment of mucus and trigger different immune gene transcription (17, 18). The immune system preserves gut homeostasis and regulates commensal microbiota via immunoglobulin A (IgA) antibodies (19). Schofield and Palm suggested that IgA shapes the gut microbiota in a similar way to how it protects against pathogens in the context of specific species growth restriction (20). Kubinak and Round observed that IgA preferentially targets, and thus limits the levels of microorganisms found in the mucosa while promoting overall microbial diversity via antibody-mediated immunoselection (AMIS), nevertheless the exact mechanisms are poorly understood (21).

Impairing IgA antibody production has been associated with reduced microbiota diversity, and imbalanced microbiota composition resulted in systemic immune activations in mice models (22, 23). One of the possible explanations is an increased opportunity for microbial translocation due to a lack of IgA, which leads to local mucosal inflammation (24). Thus, it has been hypothesized that gut microbiota might be one of the environmental drivers in CVID pathophysiology. To date, only few studies have attempted to describe gut microbiota associations with CVID or selective IgA deficiency syndrome (25–28). Jørgensen et al. showed a positive correlation between disease severity expressed by complication occurrence with higher microbiota dysbiosis and elevated immune activation markers alongside increased lipopolysaccharide (LPS) levels (26). Shulzhenko et al. observed lower mucosal IgA levels in CVID patients suffering from enteropathy than CVID patients without enteropathy and identified three different bacterial taxa that potentially contribute to CVID enteropathy (28).

These studies' results have provided a valuable insight into immunological processes occurring in CVID. However, they have not lead to a clear answer whether gut microbiota composition is causative or a consequence of a CVID phenotype, and thus further studies are needed to elucidate the true effects of low IgA levels. In addition, all studies have been focused on the microbiota's bacterial part and knowledge about fungal microbiota (mycobiota) contribution to CVID etiology is completely lacking. The mycobiota role is relevant in mediating tissue homeostasis (29, 30) and its dysbiosis has been linked to various pathological conditions as well (31, 32). Furthermore, none of the mentioned studies used patients' partners living in the same household as healthy controls to alleviate the impact of environmental cofounders on gut microbiota composition, which is very variable (33).

In this study, we attempted to expand on bacterial gut microbiota knowledge and, for the first time, fungal gut microbiota composition and its association with disease pathogenesis in CVID patients using 16S and ITS rRNA amplicon sequencing. To decrease the impact of the various environmental factors on gut microbiota composition, we also examined case-control couples living in the same household.

METHODS

This study was approved by the Ethic Committee of the Faculty of Medicine, Masaryk University (Protocol no. 37/2016). All enrolled subjects provided written informed consent.

Subject Recruitment and Sample/Data Collection

CVID patients ($n = 27$) fulfilling International Consensus Document (ICON) diagnostic criteria for CVID (3) were recruited from St. Anne's University Hospital in Brno and the University Hospital Hradec Kralove in the Czech Republic, and characterized according to Ameratunga (1) and Chapel (34) classifications (**Supplementary Table S1**). Nine patients were treated with regular intravenous immunoglobulin substitution (IVIg) and 18 with subcutaneous immunoglobulin substitution (SCIg). Five patients (18.5%) were treated with immunosuppressive medication. Twenty-two (81.5%) patients suffered from one or more of the following complications: bronchiectasis ($n = 5$), autoimmunity ($n = 9$), splenomegaly ($n = 18$), chronic diarrhea ($n = 3$), atrophic gastritis ($n = 5$), and nodular hyperplasia ($n = 2$); where 16 patients were not examined for the latter two conditions. Healthy controls ($n = 28$) included an age-, sex-, and BMI- matched cohort. Together, 27 CVID patients and 28 healthy controls formed the "ALL" group. Out of the "ALL" group, 16 healthy individuals shared the same household with 16 CVID patients as theirs partners, representing

TABLE 1 | General characteristics of study groups (ALL, PAIRS) and cohorts (CVID, CONTROLS): (a) *T*-test; (b) Fisher's exact test.

	ALL ($n = 55$)			PAIRS ($n = 32$)		
	CVID ($n = 27$)	CONTROLS ($n = 28$)	*p*-value	CVID ($n = 16$)	CONTROLS ($n = 16$)	*p*-value
Age in years	45.8 ± 12	44.8 ± 12.2	0.7505[a]	45.4 ± 11.9	44.8 ± 10.9	0.8818[a]
Mean ± SD (range)	(26–70)	(23–67)		(26–66)	(25–67)	
Male (%)	37	42.9	0.7848[b]	31.3	68.8	0.0756[b]
BMI	24.8 ± 3.7	26.7 ± 4.6	0.1032[a]	24.7 ± 4.1	26.8 ± 4.1	0.1747[a]
Mean ± SD (range)	(17.7–33.1)	(19.8–38.6)		(18.8–33.1)	(20.1–33.3)	
Smokers; Ex-smokers (*n*)	0;6	1;6	1[b]	0;4	3;1	0.1429[b]
ATB last year (>1 month) (*n*)	14	4	**0.0041[b]**	7	1	**0.0372[b]**

P-values < 0.05 (bold) are considered significant.

the "PAIRS" subgroup ($n = 32$). The clinical characteristics for the study cohorts are summarized in **Table 1**.

All participants provided self-report questionnaire data along with a fecal sample in a sterile container, according to the standardized International Human Microbiome Standards (IHMS) protocol SOP 03 V1 (35) recommended by the International Human Microbiome Consortium. Participants were excluded if they had been treated with antibiotics <1 month prior to sampling. Stool samples were accurately weighed to 200 mg aliquots and frozen at −80°C within 24 h of collection. CVID patients' IgG, IgA, and IgM serum levels were measured during routine medical visits on the day of stool sample collection.

DNA Extraction and Quantification

Fecal samples were processed using the current standard operating procedure, IHMS protocol Q (36), with minor modifications. Briefly, a frozen aliquot (200 mg) of each sample was thawed and homogenized with 0.6 g of sterile 0.1 and 0.5 mm diameter zirconia beads (BioSpec, Inc., USA) along with 1 mL ASL lysis buffer (Qiagen, Germany). Sample homogenization was undertaken on the Vortex-Genie 2 mixer (MO BIO Laboratories, Inc., USA) for 10 min and the RNase incubation step was omitted. DNA concentration and purity were determined via 260/280 and 260/230 ratios measured on the NanoDrop 1000 (Thermo Fisher Scientific, USA). DNA eluates were stored at −20°C until processing. Sterile water (B. Braun Medical, Inc., Germany) was used as no template control in each DNA extraction round ($n = 9$).

Library Preparation and Sequencing

The fecal and control samples were profiled by high-throughput amplicon sequencing using the Illumina MiSeq platform (Illumina, USA). The V3-V4 region of the bacterial *16S rRNA* gene was amplified using the primer pair (Bakt_341F/Bakt_805R) containing Illumina adapter sequences (37). Primer pairs ITS1F/ITS2 recommended by the Earth Microbiome Project[1] with unique barcode sequences designed in our laboratory (38) were used to amplify the fungal internal transcribed spacer region 1 (ITS1) of the rRNA operon. The 16S Library was constructed according to the "16S Metagenomic Sequencing Library Preparation protocol" (37). The ITS1 Library was constructed in a similar manner to the 16S Library, with minor modifications as described previously (38). As a positive control for the sequencing process, the Human Microbiome Project mock community HM-783D (obtained through BEI Resources, NIAID, NIH) also underwent PCR alongside samples and no template controls.

Bioinformatics

Sequence data analysis for both libraries was processed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (v.1.9.1.) (39). The ITS1 read pairs were demultiplexed based on the unique barcodes. Paired reads were merged and chimeric sequences were removed using VSEARCH (v. 2.6.1) (40) with the Greengenes reference database (v. 4feb2011) (41) for the 16S library and UCHIME (v. 7.2) (42) reference dataset for the ITS1 library. Chimera-free sequences were clustered into Operational Taxonomic Units (OTUs) at 97% threshold using VSEARCH *de novo*. Both OTU sets were assigned to taxonomy at 97% similarity using the Greengenes database (v. gg_13_8_otus) and Uclust (v. 1.2.22q) (43) in bacterial analysis, and BLAST (44) and UNITE (v. 7.2)[2] in fungal analysis, resulting in the OTU tables in BIOM format with the singletons discarded.

Further, sparse OTUs with a number of sequences <0.005% of the total sequence number were filtered out of the bacterial dataset (45). PyNAST (v. 1.2.2.) (46) was used to align representative sequences to build a phylogenetic tree using FastTree (v. 2.1.3) (47). QIIME was also used to calculate phylogenetic-based metrics (weighted and unweighted UniFrac distance matrices). Rare taxa with <0.01% relative abundance across all samples were excluded from the fungal OTU table using the Calypso online tool (v. 8.72) (48).

Statistical Analyses

All analyses were performed using R software (v. 3.5.2) (49) or the Calypso online tool (version 8.72) (48). OTU tables were normalized via total-sum scaling (TSS) followed by centered-log ratio transformation. All data were tested for normal distribution using the Shapiro-Wilk test for normality, and parametric or non-parametric tests were used when

[1]http://www.earthmicrobiome.org/protocols-and-standards/its/

[2]PlutoF biodiversity platform Available at: https://plutof.ut.ee/#/datacite/10.15156%2FBIO%2F587476

appropriate. The presented *p*-values were adjusted for multiple comparison corrections when appropriate. *P*-values below 0.05 were considered statistically significant.

The subjects' clinical characteristics are represented as the mean ± SD, which were determined using the *T*-test. Fisher's exact test was used to assess gender, smoking status, and previous antibiotic use differences.

Alpha-diversity expressed by the Shannon diversity, Richness and the Chao1 indices was calculated and visualized in Calypso. The Shannon diversity index measures the overall diversity (number of present OTUs, evenness), the Richness index expresses the number of present OTUs, and the Chao1 index also measures, besides OTU richness, the ratio of singletons to doubletons to give more weight to rare species (50). The *t*-test and the paired *t*-test were used to determine alpha-diversity differences between study cohorts.

Beta-diversity analyses were performed to explore associations between the microbial composition in the samples and various environmental variables via Analysis of similarities (ANOSIM), Permutational Multivariate Analysis of Variance Using Distance Matrices (ADONIS+) and Redundancy analysis (RDA+) tests were implemented in Calypso. The ANOSIM and ADONIS+ test use selected distance matrices (weighted UniFrac, unweighted UniFrac, Bray-Curtis dissimilarity). ANOSIM is a rank-based test which compares intra-group and inter-group community distances. ADONIS+ is a multivariate test which tests if the variance in microbial composition could be explained by the different explanatory variables ("ALL" and "PAIRS" groups: diagnosis, household, age, BMI, sex, ATB, and smoking status, "Patients" cohort: clinical classifications, treatment type, complication type, immunoglobulin levels). RDA+ is a supervised multivariate method which explores complex associations between microbial composition and different explanatory variables independently on a distance matrix. Selected data associations were visualized via 2D principal coordinate analysis (PCoA) plots using selected distance matrices.

Differences in microbial composition between study cohorts were identified via the: Linear discriminant analysis Effect Size (LEfSe), Differential gene expression analysis based on the negative binomial distribution (DESeq2), and regression analyses, as implemented in Calypso. LEfSe was used to identify taxa potentially associated with health status. LEfSe analysis finds taxa which are most likely to explain the differences between study cohorts. DESeq2, a test developed for count data and small sample cohorts, finds taxa which differ in their relative abundances between cohorts. Correlations between taxa and health status were assessed by regression analysis using the Spearman's correlation coefficient. Taxa which were significantly different between cohorts were visualized via stripchart plots and paired dot plots.

RESULTS

Study Population Characteristics

In this study we assigned the participants to two groups. The first main group termed "ALL" was constituted of all 55 participants enrolled in this study, 27 patients with CVID, and 28 healthy controls. The second group (a subgroup within the "ALL" group) termed "PAIRS" was consisted of 16 patients and their 16 partners from the same household, who served as controls to reduce the different environmental impacts on gut microbiota composition. The patient and control cohorts were homogeneous in age, sex, BMI, and smoking status in both groups (**Table 1**). Previous antibiotic use (more than 1 and <12 months prior to sampling) was significantly higher in the patients' cohort in both the ALL and PAIRS group (**Table 1**).

TABLE 2 | Characterization of the bacterial and fungal microbiome properties at genus level.

Genus Level (*n* = total number)	Unique (1 sample)		Frequent (> 50% samples)		Common (all samples)	
	n	%	*n*	%	*n*	%
Bacteria (*n* = 86)	0	0	67	77.9	25	29.1
Fungi (*n* = 68)	12	17.6	9	13.2	2	2.9

Unique, Number of taxa present in only one sample; Frequent, Number of taxa present in at least half of the samples; Common, Number of taxa present in all samples.

Bacterial Microbiota Differs From Fungal Microbiota

After quality-filtering steps, a total of 20,756,053 *16S rRNA* gene sequence reads and 1,789,802 *ITS1 rRNA* gene sequence reads were obtained from all 55 participants, with an average 377,383 ± 90,742 bacterial and 32,542 ± 10,768 fungal reads per sample. Reads were clustered in 552 and 163 bacterial and fungal OTUs, respectively, at 97% similarity level. Bacterial OTUs were distributed to 86 taxa, of which 63 taxa (68.2% of total reads) were assigned to the genus level. Ten OTUs from ITS1 analysis (0.42% of total reads) were not assigned to the kingdom Fungi, and the remaining 153 fungal OTUs were distributed to 68 taxa at the genus level (92.3% of total reads).

On average, 63 bacterial genera (range: 50–71) and 12 fungal genera (range: 6–21) were detected per sample. The general properties of bacterial and fungal microbiota differed. Fungal microbiota was less rich and more variable than bacterial microbiota. We detected 12 fungal genera singletons, and only two genera (2.9%) were shared by all participants, compared to 25 genera in bacterial microbiota (29.1%) shared across all samples and no singleton genus detected (**Table 2**). We detected 22 genera and 14 families in bacterial microbiota and 9 genera and 8 families in fungal microbiota with an average abundance over 1%. All taxa above a 1% average relative abundance including numbers of positive samples are listed in **Supplementary Table S2**.

Differences in Bacterial but Not Fungal Alpha-Diversity Between CVID and Controls

The alpha-diversity of the CVID and Control cohorts' gut communities in both groups was evaluated in terms of a number of observed OTUs (Richness), Shannon index and Chao1 index.

Generally, the bacterial diversity was ∼10 times higher than fungal diversity (**Table 3**), however, different sequence filtration steps were applied during analyses (see Methods). In bacterial microbiota, all measured alpha-diversity indices were lower in the CVID cohorts than in the Control cohorts, but only the difference in the Richness index was statistically significant in the ALL group ($p = 0.0256$), in contrast to the PAIRS group, where all the differences reached statistical significance (**Table 3**; **Figure 1**). In the case of fungal microbiota, alpha-diversity with health status associations were not observed in any group (**Table 3**).

Health Status and Sharing the Same Household Impact the Beta-Diversity

To identify the complex associations between the gut microbiota composition and environmental factors such as health status, same household, age, BMI, sex, antibiotic use, and smoking status, we calculated the samples' beta-diversity using the unweighted and weighted UniFrac distances (only in the bacterial analysis) and the Bray-Curtis dissimilarity distance (bacterial and fungal analyses). The Principal Coordinates Analysis (PCoA) based on Bray-Curtis measures (**Figure 2**) revealed that the CVID patients' bacterial microbiota was distinct from the healthy controls in both groups (ANOSIM, ALL: $r = 0.118$, $p = 0.001$; PAIRS: $r = 0.178$, $p = 0.001$). These observations were confirmed by multivariate tests ADONIS+ and RDA+, in which the health status was the most significant factor followed by household (PAIRS), and age (ALL) factors (**Table 4**). Similar results were obtained when using unweighted and weighted UniFrac distance matrices (**Supplementary Table S3**). Contrary to the bacterial analyses, clustering according to health status was not observed in any fungal analyses (**Figure 2**). The most significant impact on fungal microbiota composition was the same household factor (ANOSIM, PAIRS: $r = 0.47$, $p = 0.001$), as was also confirmed by ADONIS+ and RDA+ analyses (**Table 4**). Age and sex were also detected as significant factors; however, their results were inconsistently significant among analyses.

CVID Patients Harbor an Altered Bacterial but Not Fungal Gut Microbiota

To characterize the differences in bacterial and fungal microbiota abundance between CVID patients and healthy controls, we performed three different statistical tests (see Methods): The linear discriminant analysis effect size (LEfSe) (**Supplementary Table S4**), differential gene expression analysis based on the negative binomial distribution (DESeq2) (**Supplementary Table S5**), and regression analysis (**Supplementary Table S6**). Taxa were considered significantly shifted if at least two separate statistical tests discovered these taxa as biomarkers (LEfSe) or significant ($p < 0.05$; DESeq2, regression analysis). This combined analysis showed clear bacterial gut community alterations in CVID characterized by shifts in eight and 12 taxa at family and genus level, respectively (**Table 5**). These taxa were visualized by stripchart or paired dot plots of both groups

TABLE 3 | Alpha-diversity.

Diversity index	Bacteria						Fungi					
	All ($n = 55$)			Pairs ($n = 32$)			All ($n = 55$)			Pairs ($n = 32$)		
	CVID	Controls	p-value[a]	CVID	Controls	p-value[b]	CVID	Controls	p-value[a]	CVID	Controls	p-value[b]
Richness	282.3 ± 48.1	310.8 ± 43.9	**0.0256**	285.2 ± 48.6	319 ± 32.6	**0.0047**	21.3 ± 5.8	20.4 ± 6.6	**0.4531**	22.1 ± 5.9	20.6 ± 7.1	**0.63**
Shannon	3.8 ± 0.3	4 ± 0.3	**0.0621**	3.9 ± 0.3	4 ± 0.3	**0.0097**	1.1 ± 0.5	1.2 ± 0.5	**0.5829**	1.2 ± 0.4	1.1 ± 0.6	**0.56**
Chao1	336.5 ± 45.5	357.0 ± 34.2	**0.0652**	339.6 ± 41.4	361.4 ± 28.4	**0.023**	20.1 ± 6.4	21.4 ± 6.9	**0.4644**	21.1 ± 6.7	21.7 ± 7.1	**0.63**

Bacterial and fungal alpha-diversity measures characterized by the Richness, Shannon, and Chao1 indices. Mean index values ± standard deviation are shown per cohort (CVID, CONTROLS) in each group (ALL, PAIRS). P-values < 0.05 (bold) are considered significant: (a) t-test; (b) paired t-test.

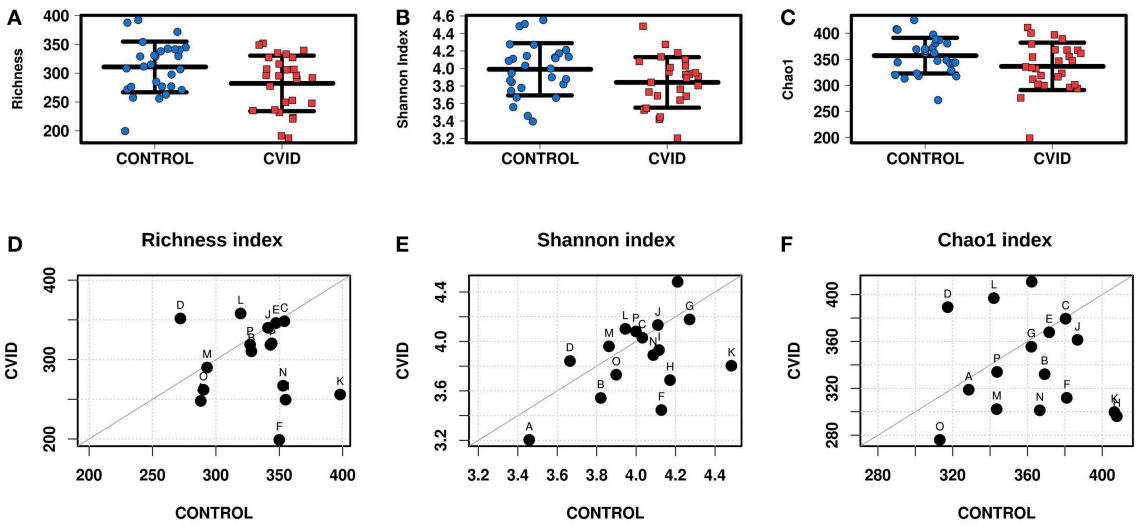

FIGURE 1 | Bacterial alpha-diversity of gut microbiome between CVID patients and healthy controls; Strip chart plots (group "ALL") and paired dot plots (group "PAIRS") depict microbiome diversity differences according to the Richness index **(A,D)**, Shannon index **(B,E)**, and Chao1 index **(C,F)**. Each letter in paired dot plots represents one pair of CVID and healthy control from the same household.

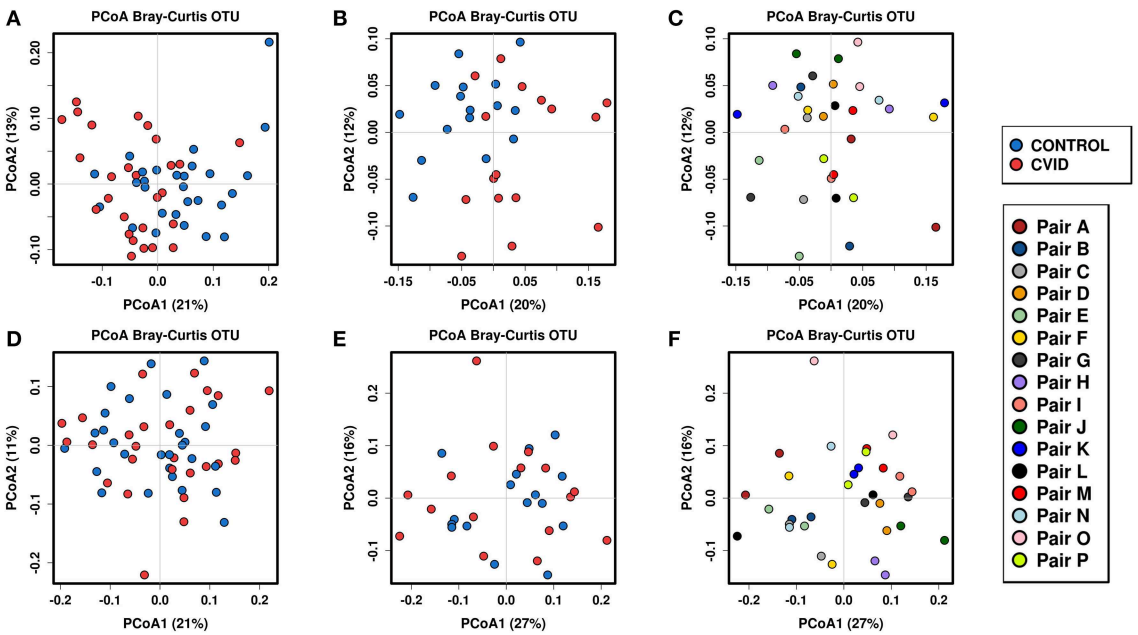

FIGURE 2 | Beta-diversity according to the health status and same household factor; Beta-diversity was calculated from total sum scaling (TSS) normalized OTU data followed by centered log-transformation ratio using the Bray-Curtis distance matrix and visualized using principal coordinate analysis (PCoA) plots. Samples are colored according to the health status (both groups), and the same household (group "PAIRS"). Bacterial beta-diversity: (i) according to the health status: group "ALL" **(A)**, group "PAIRS" **(B)**; (ii) according to the same household (group "PAIRS") **(C)**. Fungal beta-diversity: (i) according to the health status: group "ALL" **(D)**, group "PAIRS" **(E)**; (ii) according to the same household (group "PAIRS") **(F)**. Quantitative differences between cohorts are listed in **Table 4**.

(**Figure 3**). Using the same approach, we detected some shifts in the fungal composition in the context of health status (**Supplementary Tables S4–S6**); however, the results were not consistent between methods or ceased to be significant after multiple comparison correction adjustments. Only the *Blastobotrys* genus from the *Trichomonascaceae* family remained

significantly associated with CVID, although it was present in only seven samples.

More Severe CVID Phenotype and Unmeasurable Serum IgA Levels Are Associated With More Reduced Bacterial Alpha-Diversity

The CVID patient cohort ($n = 27$) was further analyzed for phenotype severity, serum immunoglobulin (IgA, IgM) levels, complication occurrence and its association with gut microbiota diversity (**Supplementary Table S1**). Patients were characterized in this study using disease classifications according to Ameratunga (1) and Chapel (34) (**Supplementary Table S1**). The CVID phenotype severity in the Ameratunga category was relatively assessed in our CVID cohort using the median as a threshold to obtain two groups—less severe ($n = 15$) and more severe ($n = 12$) phenotype. The CVID phenotypes in the Chapel category were divided into two main groups "Infection only" ($n = 15$) and "Complications" ($n = 12$) according to previously defined criteria (34).

We divided CVID cohort into two groups according to the CVID phenotype severity in the Ameratunga category and compared them with the Control cohort (**Figure 4**). We observed that less severe CVID phenotype was comparable to the controls in alpha-diversity measurements, although alpha-diversity tended to be slightly lower in CVID. Contrary to the less severe phenotype, all alpha-diversity indices in the more severe phenotype were significantly decreased than in the Control cohort (**Figures 4A–D**). CVID phenotype differences were also reflected in beta-diversity analysis (**Figure 4E**). The less severe phenotype group overlapped the Control cohort more, indicating similar microbiota composition, and the more severe phenotype tended to cluster separately, suggesting greater differences in microbiota composition. We also observed significant differences in alpha-diversity between the controls and the "Complications" subgroup assessed by Chapel category (**Supplementary Table S7**). Differences in fungal alpha-diversity associated with the mentioned classifications were not detected.

Next, bacterial alpha-diversity was lower in patients with unmeasurable serum IgA levels (<0.07 g/l) (**Supplementary Figure S1**), but only the Chao1 index indicates a significant decrease ($p = 0.015$). However, only four patients had measurable serum IgA levels in a sampling day.

We did not observe any other conclusive differences in bacterial or fungal alpha-diversity in a complication occurrence context (bronchiectasis, autoimmunity, splenomegaly, chronic diarrhea, atrophic gastritis, and nodular hyperplasia) (**Supplementary Table S8**), treatment administration (antibiotic use, substitution therapy type or immunosuppression), or serum IgM levels, however, data analyses in most groups/categories may be burdened by a small number error. Therefore, interpreting the results of these analyses is difficult and more samples would be needed to analyze these complications to resolve whether gut microbiota is affected or not.

DISCUSSION

In this study, we attempted to: (i) Assess whether the bacterial and/or fungal gut microbiota is affected in CVID by comparing the gut microbiota composition between CVID patients and healthy control cohorts in two groups constituting all participants and case-control pairs who shared the same household, respectively; and (ii) Expand general knowledge about fungal gut microbiota composition, since gut fungi are still relatively understudied and new findings are needed regardless of the study's primary purpose. To achieve these goals, we used alpha- and beta- diversity measurements alongside taxonomic comparisons. Alpha-diversity shows how many and how many different microbes can be found within one sample. Beta-diversity shows how these samples or groups of samples (i.e., CVID vs. Controls) vary against each other, and taxonomic comparisons specifically show these differences.

Our results indicate that CVID patients harbor less diverse and significantly altered bacterial communities in their gut. It also appears that the gut bacterial microbiota is associated with the CVID phenotype severity, which is in concordance with the previous study's outcome that bacterial microbiota may be involved, at least partially, in systemic immune activation in CVID (26). Contrary to bacterial analyses, only one relatively underrepresented fungal genus *Blastobotrys* was associated with CVID in our study, however, this genus was present in only a few samples, and thus mycobiota statistical analyses exhibited no conclusive health status associations indicating that fungi are probably not relevant contributors to the CVID phenotype. In parallel, we examined a group of 16 case-control couples who shared the same household to decrease the different environmental impact on gut microbiota composition. We found that sharing the same household is a strong factor influencing the microbiome data. While in the case of bacterial analyses, the household's impact was strong but did not outweigh the influence of the health status on microbiota diversity, in the case of fungal analyses, the household was the most significant diversity determining factor.

Gut Microbiota in CVID

In last few decades we have expanded our understanding of the role of the human gut microbiome in health and disease. Many studies associated gut microbiomes not only with gastrointestinal tract diseases, but also with extra-intestinal conditions including immune system disorders [reviewed in (51)]. Nevertheless, studies linking the gut microbiome to CVID are still rare. To the best of our knowledge, only three studies have focused on gut microbiota composition in CVID patients (25, 26, 28), and none of them analyzed gut mycobiota or used paired controls from others sharing the same household. We provide a new view on CVID bacterial microbiota alongside previous findings' comparisons, and at the same time provide unique findings from CVID gut mycobiota analysis. Our results from bacterial analyses could be most easily compared to Jørgensen et al. (26) study results since other two mentioned studies (i) used different

TABLE 4 | Bacterial and fungal beta-diversity by environmental variables.

Parameter	ADONIS+ Bray-Curtis				Anosim Bray-Curtis				RDA+					
	All ($n = 55$)		Pairs ($n = 32$)		All ($n = 55$)		Pairs ($n = 32$)		All ($n = 55$)			Pairs ($n = 32$)		
	R^2	*P*	R^2	*P*	*R*	*P*	*R*	*P*	Variance	*F*	*P*	Variance	*F*	*P*
BACTERIA														
Diagnosis	0.0665	**0.0003**	0.0862	**0.0003**	0.118	**0.001**	0.178	**0.001**	20.50	2.11	**0.001**	30.43	1.86	**0.003**
Household	ND	ND	0.585	**0.0027**	ND	ND	0.352	**0.002**	ND	ND	ND	291.10	1.19	**0.03**
Age category	0.109	**0.006**	0.0887	0.63	0.118	**0.004**	0.156	**0.018**	51.44	1.32	**0.004**	60.46	0.92	0.729
BMI category	0.0608	0.146	0.0402	0.726	0.064	0.077	−0.021	0.609	33.34	1.14	0.149	29.88	0.91	0.716
Sex	0.0133	0.669	0.0221	0.555	−0.008	0.541	0.036	0.139	8.55	0.88	0.772	14.30	0.87	0.742
ATB	0.0166	0.414	0.0159	0.868	0.04	0.234	0.041	0.335	9.23	0.95	0.570	13.99	0.85	0.765
Smoking	0.0396	0.201	0.0421	0.663	0.041	0.333	0.097	0.213	20.61	1.6	0.283	30.3	0.92	0.711
FUNGI														
Diagnosis	0.0213	0.29	0.0173	0.528	0.004	0.341	−0.031	0.851	3.10	1.00	0.426	3.73	0.79	0.760
Household	ND	ND	0.701	**0.0007**	ND	ND	0.47	**0.001**	ND	ND	ND	97.05	1.37	**0.033**
Age category	0.102	0.052	0.122	0.0733	0.047	0.096	0.14	**0.04**	15.90	1.29	**0.003**	23.38	1.24	0.220
BMI category	0.0417	0.818	0.0375	0.501	0	0.504	0.032	0.283	11.68	1.26	0.175	7.15	0.76	0.833
Sex	0.0205	0.318	0.0123	0.78	−0.086	0.996	−0.031	0.841	3.43	1.11	0.223	10.11	2.15	**0.021**
ATB	0.0059	0.984	0.0041	0.993	−0.005	0.517	0.042	0.318	2.40	0.78	0.906	2.41	0.51	0.965
Smoking	0.0391	0.415	0.0082	1	−0.08	0.775	−0.097	0.779	5.68	0.92	0.657	4.62	0.49	1

Beta-diversity was calculated from total-sum scaling (TSS) normalized OTU data followed by centered-log ratio transformation using the Brays-Curtis dissimilarity distance matrix. Analysis of variance using distance matrix (ADONIS+) adjusted for multiple variables: R^2 value indicates effect size. Analysis of similarities (ANOSIM): R is constrained between the values −1 to 1, where positive numbers suggest more similarity within sites, negative numbers more similarity between sites and values close to zero represent no differences. Bray-Curtis distance independent redundancy analysis (RDA+) adjusted for multiple variables. P-values below 0.05 (bold) were considered significant.

TABLE 5 | Bacterial taxa at genus and family level significantly associated with health status across at least two of three statistical tests.

Selected taxa	Associated group	LEfSe LDA score	DESeq2 p-value (FDR)	Regression analysis *p*-value
FAMILY LEVEL				
Campylobacteraceae	CVID	3.09	0.0000000013	0.0013
Streptococcaceae	CVID	3.96	0.0000021	0.0074
Lactobacillaceae	CVID	NS	0.00011	0.05
Gemellaceae	CVID	2.95	0.00014	0.00078
Unclassified.Lactobacillales	CVID	NS	0.013	0.0097
Enterobacteriaceae	CVID	3.68	NS	0.0089
Erysipelotrichaceae	CONTROL	3.73	0.019	0.031
Unclassified_Clostridiales	CONTROL	4.12	0.042	0.01
GENUS LEVEL				
Campylobacter	CVID	2.27	0.0000000063	0.0012
Anaerotruncus	CVID	2.44	0.0000044	0.00086
Streptococcus	CVID	3.95	0.0000063	0.012
Gemella	CVID	2.29	0.00052	0.00096
Lactobacillus	CVID	NS	0.00073	0.038
Eggerthella	CVID	2.61	0.0047	0.0013
Unclassified.Lactobacillales	CVID	NS	0.0047	0.0092
Enterococcus	CVID	NS	0.0047	0.02
Unclassified_Enterobacteriaceae	CVID	3.72	NS	0.0015
Mitsuokella	CONTROL	3.02	0.00014	NS
Unclassified_Clostridiales	CONTROL	4.11	0.0039	0.0092
Unclassified_Coriobacteriaceae	CONTROL	3.22	0.026	0.007

Taxa are listed by the DESeq2 significance in each cohort. LEfSe, The linear discriminant analysis (LDA) effect size; DESeq2, Differential gene expression analysis based on the negative binomial distribution; NS, Not significant.

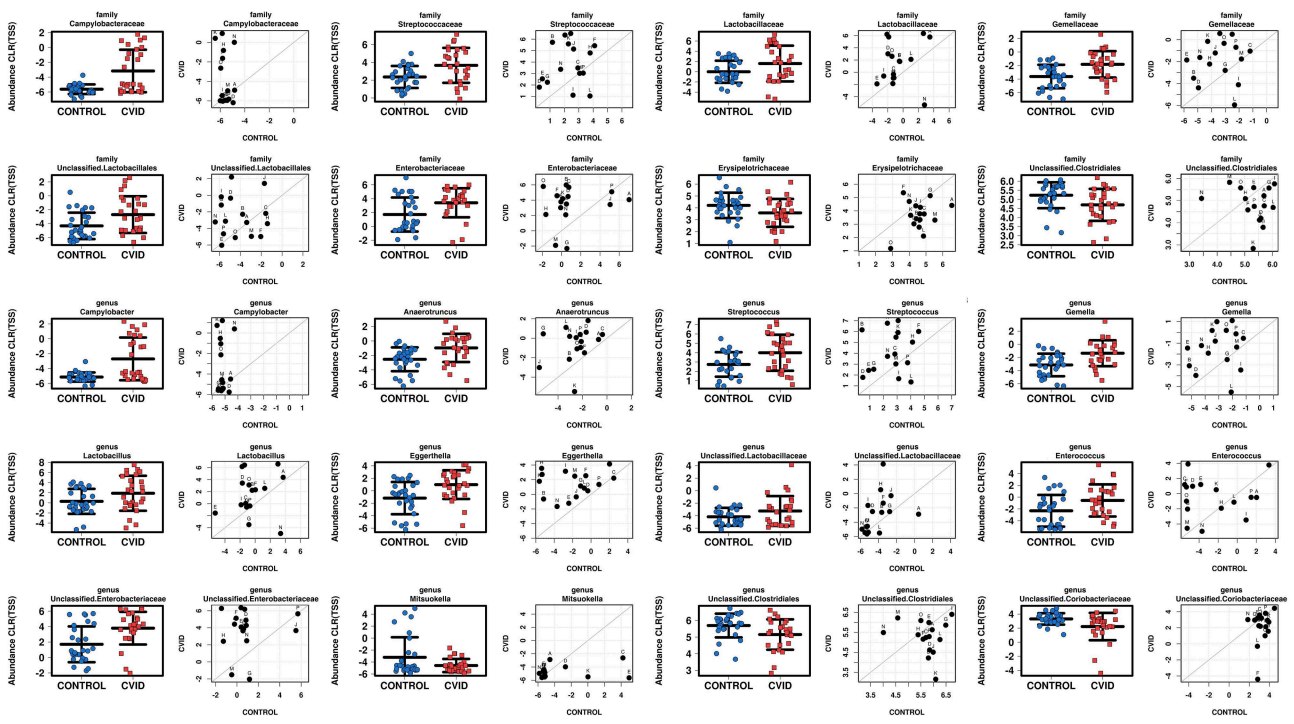

FIGURE 3 | Comparison of total sum scaling (TSS) and centered log-ratio transformation data from bacterial taxa according to health status in group "ALL" (stripchart plots) and group "PAIRS" (paired dot plots). Figure shows 8 and 12 taxa at family and genus level, respectively, significantly altered in CVID patients (**Table 5**).

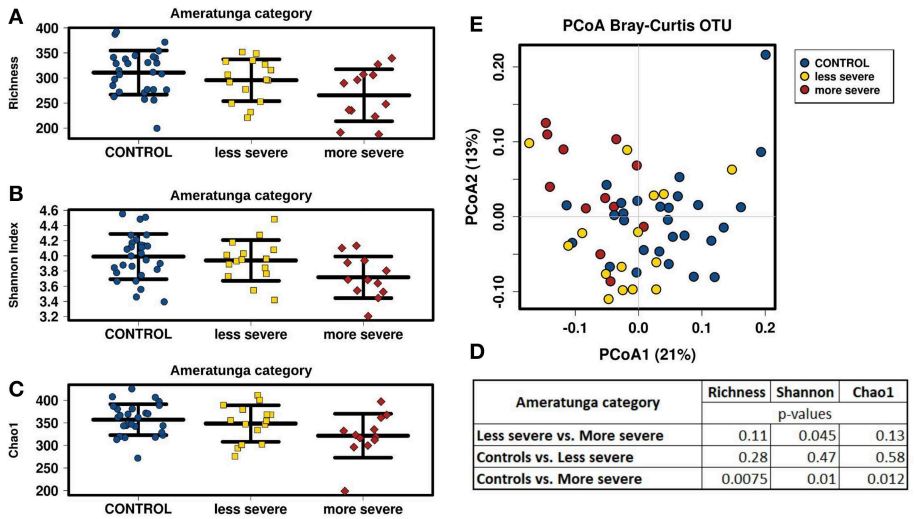

FIGURE 4 | CVID phenotype severity comparison; CVID patients with less severe ($n = 15$) and more severe ($n = 12$) disease phenotype assessed by "Ameratunga category" (**Supplementary Table S1**) were compared to Control cohort. Stripchart plots depict bacterial microbiome diversity differences according to the Richness index **(A)**, Shannon index **(B)**, and Chao1 index **(C)**. *P*-values were calculated using t-test **(D)**. Principal coordinate analysis (PCoA) plot of beta-diversity depict clustering according to the health status and CVID phenotype severity **(E)**.

methodology and/or sample type, (ii) analyzed a small number of CVID patients, and (iii) did not report the full list of CVID taxa abundance for comparison.

Bacterial Gut Microbiota in CVID

In agreement with previous findings (25, 26), we observed reduced bacterial diversity in CVID patients. Recently reduced bacterial alpha-diversity was recognized as a possible universal gut microbial biomarker of common human intestinal diseases (52). The main idea of the hypothesis that higher microbiota diversity is connected with better health status lies in the greater ability to adapt to possible perturbations. However, diversity alone may be a poor marker of disease (53), and sometimes "the higher microbiota diversity the better" hypothesis does not hold true (54). On the other hand, low diversity combined with microbial dysbiosis might have a biological significance (53). We detected perturbations in taxa abundance in CVID patients, also reflected the in the beta-diversity analyses, where the CVID cohort tended to cluster separately from the Control cohort. For example, increased *Streptococcaceae*, *Lactobacillaceae,* and *Enterobacteriaceae* families in our CVID cohort alongside reduced diversity have been linked to other diseases inside (52), and also outside (55) the gut, suggesting the existence of the common dysbiosis feature (56). It is also noteworthy to mention that human-pathogenic genus *Campylobacter* was exclusively present in our seven CVID patients with no GIT symptoms reported suggesting their different susceptibility to *Campylobacter* colonization and the inability of their immune system to adequately respond to it (57).

We also suggest that reduced diversity in CVID patients in our dataset has biological significance, since patients with a more severe CVID phenotype displayed more decreased alpha-diversity than patients with a less severe CVID phenotype. CVID patients also clustered more distantly in beta-diversity analyses, which indicates different microbiota composition. Next, alpha-diversity was also lower in patients with unmeasurable serum IgA levels; however, as low IgA levels are typical for CVID, there were only four with measurable serum IgA for comparison in our group. These results partially correspond with results of Jørgensen et al. (26), where CVID patients with complications, and/or with decreased plasma IgA levels also had reduced alpha-diversity when compared with patients suffering from infections only, and/or with normal plasma IgA levels. However, plasma/serum IgA levels do not necessarily correspond with secretory IgA levels, which are known to impact CVID gut microbiota directly (28). In their study, Shulzhenko et al. observed that CVID patients with low levels of secretory IgA developed CVID enteropathy whereas patients with normal secretory IgA levels did not. Despite the secretory IgA antibodies being polyreactive, they are only able to coat a restricted spectrum of microbes (58). Species known to be IgA-coated mainly include members of the *Proteobacteria* phylum (i.e., *Enterobacteriaceae*) and *Firmicutes* phylum (i.e., *Lactobacilli*) (19). These members are also relatively elevated in our CVID cohort, and it can be speculated that the "CVID dysbiosis" may be associated with depleted secretory IgA. Nevertheless, these results should be verified in larger patient cohorts with known plasma/serum and secretory IgA levels. Furthermore, it has been very recently described that systemic IgG and secretory IgA bind a common spectrum of gut microbiota, therefore secretory IgA depletion alone may often remain asymptomatic since IgG may provide a second level of protection (59). However, IgG protection seems to be personalized, and although immunoglobulin replacement therapy administered to CVID patients contains an extended set of anti-commensal IgG, it seems to bind CVID microbiota less

efficiently, and thus may lead to microbiota dysregulation and possible dysbiosis (59).

We further compared all taxa significantly altered in our study to the CVID microbiota results from other studies (25, 26, 28). Our main results were mostly in agreement with these studies; however, some contradictories were detected. First, Shulzhenko et al. (28) did not observe any significant differences in bacterial abundance between CVID and controls, which is in contrast to our results; however, a low number of duodenal biopsies had been evaluated in their study so the results are not easily comparable. Second, Fadlallah et al. (25) described low *Actinobacteria* phylum diversity in their CVID patients with very low secretory IgM levels, which was also not observed in our study, however, we did not measure intestinal immunoglobulin levels; therefore we cannot exclude CVID phenotype differences' impact in our cohorts. Last, Jørgensen et al. (26) calculated a "CVID specific dysbiosis index" using several taxa significantly altered in their CVID cohort. These taxa did not differ from the healthy control group in our study except taxa from *Bacilli* class and *Enterobacteriaceae* family. Furthermore, we even detected *Anaerotruncus* and *Eggerthella* genera to be significantly increased in CVID, which was opposite to their results. Therefore, we suppose that the taxa differences may not be strongly associated with the CVID phenotype, or other confounding factors may have influenced the resulting outcome such as (i) geographical origin, (ii) different CVID spectrum analyzed, and (iii) other factors including methods and cohort size differences.

In parallel, within our participant group, we analyzed 16 CVID patient-partner pairs sharing the same household to diminish the impact of different environmental factors. We confirmed that most pairs were more similar to each other than to the strangers in terms of bacterial microbiota composition, which corresponds to previous findings (60, 61). However, despite the similarities between the pairs, the health status remained the most significant feature in beta-diversity analyses. Furthermore, health status differences become even more visible in alpha-diversity analyses as well as in taxa abundance analyses when performing paired tests (see Methods). These results indicate that despite the high inter-individual and inter-pair variability, the CVID phenotype was well reflected in the bacterial data.

Fungal Gut Microbiota Properties

Fungal gut microbiota research lags way behind those for bacteria, although it has recently attracted more attention. One of the Human Gut Microbiome Project (HMP) studies has widened our knowledge about "healthy" fungal gut microbiota (62), and there are many other studies correlating gut mycobiota composition and its role in various health issues and disease conditions [reviewed in (63)]. There, gut fungi were characterized as low in diversity and very variable between individuals (62, 64, 65). In line with previous findings, we observed a higher fungal variability between samples and ∼10 times lower fungal diversity than the bacterial analyses in our study. On average, one participant harbored 12 fungal genera; however, only a few fungal genera were shared by more than a third of participants, and these genera represented a core mycobiome in this study. The most abundant core mycobiome genera were identified as *Saccharomyces*, *Penicillium*, *Dipodascus*, *Debaryomyces*, *Candida*, *Pichia*, *Aspergillus*, *Rhodotorula*, and *Hanseniaspora*. The mentioned fungal genera were also found in various abundance in the stool samples by others (62, 64, 66), however, some genera such as *Malassezia* or *Cryptococcus*, commonly detected elsewhere (62, 67), were not present in our samples. Inconsistencies in results may be partially explained by the different methodology, such as different fungal primers used across the studies. For example, primer pair targeting the ITS1 region, which was used in this study, is known to underrepresent *Malassezia* genus abundance (68). The current nomenclatural changes implemented in the UNITE database (69, 70) provide another explanation and affect many fungal taxa including *Cryptococcus* genus, which was taxonomically assigned to its homotypic synonyms (*Filobasidium*, *Vishniacozyma*, *Naganishia*, *Filobasidium*, and *Cutaneotrichosporon*) in our data. Moreover, geographical-based differences might also impact the results similarly as described in bacterial gut microbiota research (71).

Fungal Gut Microbiota in CVID

To the best of our knowledge, this is the first study analyzing gut mycobiota in CVID patients; therefore, there is no other similar dataset for an appropriate comparison. First, we report no differences in fungal alpha-diversity between patients and controls; however, the impact of fungal alpha-diversity on human health is still unclear. Overall fungal alpha-diversity was ∼10 times lower than bacterial alpha-diversity, and contradictories in the same disease-related alpha-diversity measurements were reported (29, 72), suggesting that fungal alpha-diversity may not be critical in disease evaluation. Second, we did not observe any obvious taxonomic differences between patients and controls in our study, indicating that gut mycobiota may not affect the CVID phenotype, at least not in a detectable way. Nevertheless, we observed one fungal CVID phenotype association, specifically *Blastobotrys* genus, although it was present in only six CVID and one control sample. This genus was not previously reported in the context of the human gut, and therefore it may not be a true gut colonizer. On the other hand, *Blastobotrys* species are capable of growing in 37°C, and they were reported to cause invasive fungal infections, although extremely rare, in immunocompromised patients (73). Therefore, this possible association deserves deeper examination. All in all, we did not find any convincing associations between gut mycobiota and CVID phenotype, however, since the properties of fungal composition differ from bacterial composition tremendously, as described above, we cannot exclude the fungal impact may lie in other aspects than in taxa diversity or abundance differences. For example, various fungal species overgrowth, which was not measured in our study since only taxa relative abundance was assessed, has been often found to correlate with disease state in other similar mycobiota studies (29), and thus our study may not have revealed all possible associations.

Similar to bacterial analyses, we further evaluated a smaller "PAIRS" group in terms of mycobiota diversity, and we did not observe any health status associations in this dataset, which corresponds with the results stated above. Instead, most of the paired samples clustered close to each other in beta-diversity analyses, indicating a similar fungal mycobiota composition in members of the same household. It agrees with the correlative associations observed both in a mice study, where mice from the same cage were colonized with specific fungi differing from other cages (74), and in only one human study, focusing on human mycobiota transfer from mother to offspring (75). Other data from human research are unfortunately lacking, therefore the biological relevance of these correlations remains unknown. Thus, future studies using paired samples from the same household could provide a new research opportunity to assess whether gut mycobiota is truly capable of colonizing human gut and can be transferable between partners, or is only the transiently present fungal DNA originating from the environment, as was previously outlined elsewhere (76).

CONCLUSION

In summary, our study extends previous findings of the correlation between the bacterial gut microbiota and CVID and provides new insights into overall gut mycobiota composition. Furthermore, we reveal the strong impact of sharing the same household on bacterial and fungal microbiome data, although weaker than that of CVID diagnosis in bacterial assessment. This suggests that paired samples serving as controls in future studies would provide a better resolution between disease-related dysbiosis and other environmental confounders. The cause of CVID still remains unknown in most cases; however, gut microbiota may be one of the missing environmental drivers contributing to some of the symptoms and their severity. Although, due to the high CVID heterogeneity and the limits of current methodology, it is still unclear whether CVID results in dysbiosis and/or dysbiosis contributes to the CVID phenotype. Therefore, the larger metacentric studies including gut microbiota profiles, metagenomics, metabolomics, as well as more detailed and topical immunological evaluation will be needed to further establish the relevance of gut microbiota in CVID.

DATA AVAILABILITY

The raw sequences of datasets for this study can be found in the European Nucleotide Archive repository, http://www.ebi.ac.uk/ena under accession number: PRJEB32265.

AUTHOR CONTRIBUTIONS

TF, DS, JL, KF, and JB conceived the initial project design and discussed project progress. IK provided partial assistance with experimental project workflow. KF, MC, and EN processed stool samples. KF performed all experiments and statistical analyses of the data with significant contributions from EN and HG. MR performed the bioinformatics analysis of the data. ML provided bioinformatics assistance. PK and JL provided stool samples alongside patients' clinical and laboratory characteristics. KF, HG, and TF wrote the manuscript and EN, JB, DS, IK, and JL significantly contributed to the final preparation of the manuscript. All authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01914/full#supplementary-material

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CASE REPORT
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A Novel *BTK* Gene Mutation in a Child With Atypical X-Linked Agammaglobulinemia and Recurrent Hemophagocytosis: A Case Report

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X-linked agammaglobulinemia (XLA), caused by a mutation in the Bruton's tyrosine kinase (*BTK*) gene, is rarely reported in patients with recurrent hemophagocytic lymphohistiocytosis (HLH). This mutation leads to significantly reduced numbers of circulatory B cells and serum immunoglobulins in patients. Therefore, they exhibit repetitive bacterial infections since infancy, and immunoglobulin (Ig) replacement therapy is the primary treatment. HLH is a life-threatening condition with manifestations of non-remitting fever, hepatosplenomegaly, cytopenias, coagulopathy, lipid disorder, and multiple organ failure. It is caused by the immune dysregulation between cytotoxic T cells, NK cells, and histiocytes. The treatment is based on HLH-2004 protocol including immunotherapy, chemotherapy, supportive therapy, and stem cell transplantation. However, as we know more about the classification and pathophysiology of HLH, the treatment is modified. T-cell-directed immunotherapy is effective in patients with primary HLH, and strong immunosuppression is contraindicated in patients with severe ongoing infections or some primary immunodeficiency diseases (PIDs). Here, we report the case of a 7-year-old boy who presented with ecthyma gangrenosum and several episodes of pyogenic infections during childhood. At the age of 5 years, he exhibited cyclic HLH every 2–3 months. The remission of HLH episodes finally achieved after he received monthly Ig replacement therapy (400 mg/kg) at the 4th HLH. However, transient elevation of IgM was incidentally discovered after 6 cycles of monthly Ig replacement therapy. IgM-secreting multiple myeloma, Waldenström's macroglobulinemia, and lymphoma were excluded. The IgM levels then declined and returned to the normal range within a year. The patient and his parents received whole-genome sequencing analysis. It revealed a novel hemizygous c.1632-1G>A mutation in the *BTK* gene and XLA was diagnosed. XLA exhibits a spectrum of clinical and immunological presentations in patients. The identification of the mutation in the *BTK* gene contribute to an accurate diagnosis. Ig replacement therapy is the primary treatment for HLH in patients with XLA.

Keywords: Bruton's tyrosine kinase, hemophagocytic lymphohistiocytosis, intravenous immunoglobulin, primary immunodeficiency disease, X-linked agammaglobulinemia

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INTRODUCTION

Background

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disease (PID) characterized by hypogammaglobulinemia with a small number of peripheral circulating mature B cells (<1%) in a male patient (1). It is caused by a mutation in the Bruton's tyrosine kinase (*BTK*) gene that leads to the failure of B cells to develop from the pro-B to pre-B stage. Patients present with frequent bacterial infections from the age of 6 months as their mothers' protective transplacental immunoglobulin (Ig) G depletes. From early childhood, they exhibit recurrent respiratory infections such as sinusitis, pneumonia, and otitis media; severe bacterial infections such as septicemia, osteomyelitis, and meningitis may also occur. They can fight most viral infections well because of the preserved number and function of T cells, but they are susceptible to hepatitis viruses and some enteroviruses. XLA is associated with some autoimmune diseases, malignancy, and growth hormone deficiency (2). It is safe and effective to treat patients with XLA with immunoglobulin (Ig) replacement therapy 400–600 mg/kg every 3–4 weeks to prevent severe infections (3). Hematopoietic stem cell transplantation in this patient group has not been fully studied and is not yet recommended, unless the patient presents with a malignant hematologic disease (4).

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening hyperinflammation caused by immune dysregulation between cytotoxic T cells, NK cells, and histiocytes, resulting in non-remitting fever, hepatosplenomegaly, cytopenias, coagulopathy, lipid disorder, and organ infiltration by activated macrophages performing phagocytosis (5, 6). Primary HLH is caused by highly activated T cells with defective cytotoxicity. Patients with primary HLH have genetic mutations related to: (1) granule-mediated cytotoxic T and NK cells that result in familial HLH (*PRF1*, *UNC13D*, *STXBP2*, *STX11*); (2) lysozyme function that results in Chédiak–Higashi syndrome (*LYST*), Griscelli syndrome type 2 (*RAB27A*), and Hermansky–Pudlak syndrome 2 (*AP3B1*); and (3) the inhibited cytotoxic responses of cytotoxic T and NK cells to B cells infected by Epstein–Barr virus (EBV) that result in X-linked lymphoproliferative syndrome (*SH2D1A*, *XIAP*). Though rarely occurring, other PIDs have been reported to cause HLH, including combined immunodeficiency, chronic granulomatous disease, autoinflammatory diseases, and antibody deficiencies (5–9). In addition, a viral infection may trigger a flare-up in a patient with primary HLH. More Taiwanese patients with HLH have a disease type relevant to EBV infection (10). Secondary HLH, also known as macrophage activation syndrome, is present in patients undergoing severe infections, malignancies, autoimmune, or autoinflammatory diseases (7–10). It is challenging to distinguish between primary and secondary HLH by the initial presentations of a patient. The treatment is based on HLH-2004 protocol, including chemoimmunotherapy (corticosteroids, etoposide, cyclosporin A, and/or intrathecal methotrexate), prophylactic antibiotics, and intravenous Ig (IVIg). Hematopoietic stem cell transplantation is indicated in selected patients with refractory and/or relapsed disease after appropriate chemoimmunotherapy (11–13). However, it may be inappropriate and harmful to treat with aggressive immunosuppression in the patients with severe ongoing infections or PIDs other than familial HLH and X-linked lymphoproliferative syndrome. Furthermore, more T-cell targeting therapies, such as anti-thymocyte globulin, etoposide, and alemtuzumab, can be effective in patients with primary HLH caused by the overactivated T cells (8, 9).

Case Presentation

This 7-year-old boy suffered his first severe infection when he was aged 1 year, presenting with fever, vomiting, and diarrhea for 2 days, followed by the appearance of multiple enlarged erythematous rashes on the head, trunk, and limbs. The skin rash in his lower legs progressed, and some lesions became necrosis (**Figure 1**). The diagnosis of ecthyma gangrenosum was established on the basis of all available cultures yielding wild-type *Pseudomonas aeruginosa*. He also had septic shock; therefore, he received fluid resuscitation, meropenem administration, inotropic agents infusion, and endotracheal tube insertion with mechanical ventilation at intensive care unit. The necrotic wounds gradually improved and became scarred. Since then, he has had bacteremia, pyogenic pneumonia, sinusitis, and osteomyelitis several times. At the age of 5 years, he experienced 4 episodes of HLH over a period of 9 months that presented as persistent fever; hepatosplenomegaly; pancytopenia; disseminated intravascular coagulation; and increased levels of triglycerides, ferritin, and soluble interleukin-2 receptor. Moreover, he had chronic hepatitis B in the immune tolerance stage for years under lamivudine treatment. The patient's family medical history included the death of his mother's brother in infancy from an unknown disease and his mother being a hepatitis B virus carrier. Physical examination indicated

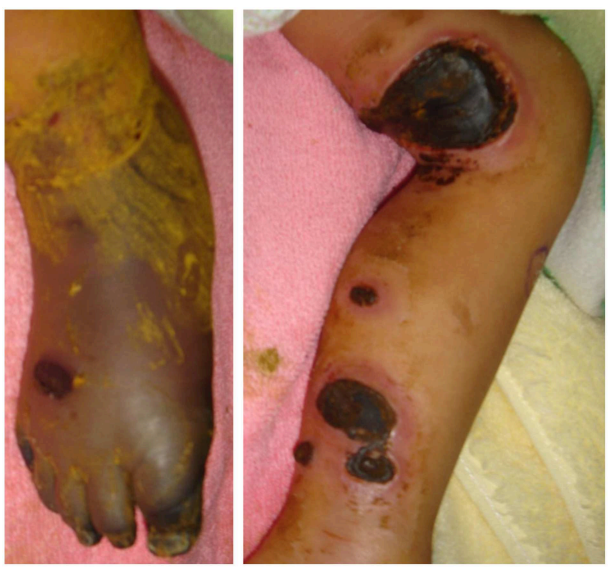

FIGURE 1 | The ecthyma gangrenosum with poor peripheral perfusion over the patient's bilateral legs.

TABLE 1 | The criteria and investigations of the index case for hemophagocytic lymphohistiocytosis and X-linked agammaglobulinemia.

Hemophagocytic lymphohistiocytosis (HLH)									**X-linked agammaglobulinemia (XLA)**	
At least 5 of the following 8 findings: **1. Non-remitting fever ≥38.5°C** **2. Splenomegaly** **3. Cytopenia: Hb ≤ 9 g/dL, Plt ≤ 100 × 1,000/μL, ANC ≤ 1,000/μL (at least 2)** **4. Hypofibrinogenemia (<150 mg/dL) or hypertriglyceridemia (≥265 mg/dL)**						**5. Hyperferritinemia (≥500 ng/mL)** **6. Increased level of soluble CD25 (sIL-2R)** **7. Evidence of hemophagocytosis in BM, LN, spleen, or liver** **8. Decreased or absent NK cell cytotoxicity.**			**Hypogammaglobulinemia and near absence of peripheral circulating B cells**	
	Fever	**Splenomegaly**	**Cytopenia**	**Fibrinogen (mg/dL)**	**TG (mg/dL)**	**Ferritin (ng/mL)**	**sIL-2R[a] (pg/mL)**	**Hemophagocytosis**	**Other survey**	**Ig levels (mg/dL)**	**Lymphocyte subset**
1st HLH	+	+	+	95.2	344	1,214	>5,000	Not performed	EB VCA-IgG: 1.142[b] EB VCA-IgM, EB-NA Ab, CMV-IgM, CMV-IgG: Neg.		
2nd HLH	+	+	+	80.9	270	791	>5,000	BM	BM culture: No pathogen growing.	IgA: < 10.46[c] IgG: 639[c] IgM: < 1.09[c]	
3rd HLH	+	+	+	131	213	690	4,263	Not performed		IgA: 18.1 IgG: 236 IgM: 7.9	T-cell: 98.52%, B-cell: ND, NK-cell: 1.48%
4th HLH	+	+	+	81.3	308	503	3,322	Not performed	Viral DNA PCR of HSV-1, VZV, EBV, CMV, parvovirus B19: Neg.	IgA: <10.46 IgG: 259 IgM: <1.09	T-cell: 99.98%, B-cell: 0.02%, NK-cell: N/A

+, present; [a]Negative, < 880 pg/mL; [b]Cuff-off-value, 1.1; [c]The data were checked 1 month after the infusion of immunoglobulin, 1 g/kg/day for 2 days, at the 2nd HLH, ANC, absolute neutrophil count; BM, bone marrow; CMV, cytomegalovirus; EBV, Epstein–Barr virus; EB VCA-IgG, Epstein–Barr virus viral capsid antigen immunoglobin G; EB VCA-IgM, Epstein–Barr virus viral capsid antigen immunoglobin M; EB-NA Ab, Epstein–Barr virus nuclear antigen antibody; Hb, hemoglobin; HLH, Hemophagocytic lymphohistiocytosis; HSV, herpes simplex virus; Ig, immunoglobulin; LN, lymph node; N/A, Not available; ND, Non-detectable; Neg, Negative; PCR, polymerase chain reaction; Plt, platelet; TG, triglyceride; sIL-2R, soluble interleukin-2 receptor; VZV, varicella zoster virus.

the patient had failure to thrive, normal hair, intact skin except previous wound scars, a faint Bacillus Calmette–Guérin vaccine scar, invisible tonsils, and hepatosplenomegaly. His laboratory data were summarized in **Table 1**. The first HLH episode subsided soon after the infusion of IVIg (1 g/kg/day for 2 days). He did not undergo chemotherapy, as per the HLH protocol. He received oral prednisolone 2 mg/kg/day for 28 days followed by a gradual reduction in prednisolone dosage within 21 days. However, HLH recurred 1 month later after prednisolone was discontinued. The report from the bone marrow biopsy detailed non-caseating granulomatous inflammation with hemophagocytosis and the absence of a pathogen growing in the culture of the bone marrow. The second HLH subsided under IVIg therapy (1 g/kg/day for 2 days). He also received a 28-day course of oral prednisolone (2 mg/Kg/day) and then a 21-day course of prednisolone tapering. He was then kept on a low dose of prednisolone 0.25 mg/Kg every day for 3 weeks. The third HLH still recurred, and it also subsided quickly after IVIg therapy (1 g/kg/day for 2 days). He took prednisolone 2 mg/Kg/day for 14 days, with dose tapering within the next 28 days. Then he had the 4th episode of HLH around 1 month later. The first available data of Ig levels, checked 1 month after the infusion of IVIg (1 g/kg/day for 2 days) at the second episode of HLH, indicated the following: IgG: 639 mg/dL, IgA < 10.46 mg/dL, IgM < 1.09 mg/dL. The Ig data at the third episode of HLH before the commencement of IVIg infusion showed IgG: 236 mg/dL, IgA 18.1 mg/dL, and IgM 7.9 mg/dL, and the lymphocyte subsets exhibited no detectable B cells. The follow up data at the fourth episode of HLH were as follows: IgG: 259 mg/dL, IgA <10.46 mg/dL, and IgM <1.09 mg/dL with very few B cells (0.02%). The viral DNA PCR of herpes simplex virus-1, varicella zoster virus, EBV, cytomegalovirus, and parvovirus B19 were checked at the 4th episode of HLH showed negative results.

Considering the repeated bacterial infections experienced by the patient since infancy, as well as the recurrent episodes of HLH, global hypoglobulinemia, and the depletion of circulatory B cells, a PID entailing a B-cell defect, especially an X-linked defect, was highly suspected. The patient started regular Ig replacement therapy (400 mg/kg) every 4 weeks without receiving prednisolone after the 4th episode of HLH, which prevented further development of HLH attacks or the severe infections. After a 6-month course of monthly replacement of Ig (400 mg/kg), a significantly increased level of IgM (1,273 mg/dL) was discovered during a regular blood check-up. An IgM kappa monoclonal band was found in his serum immunofixation exam **Table S1** in Supplementary Material. We performed whole genome sequencing analysis of the patient and both his parents. Genetic analysis revealed a novel hemizygous c.1632-1G>A mutation in the *BTK* gene of the patient (**Figure 2**). He was diagnosed to have XLA, and his mother is the carrier.

Method

Whole-genome sequencing was performed using Illumina Novaseq 6000 System (Illumina Inc., San Diego, CA, USA).

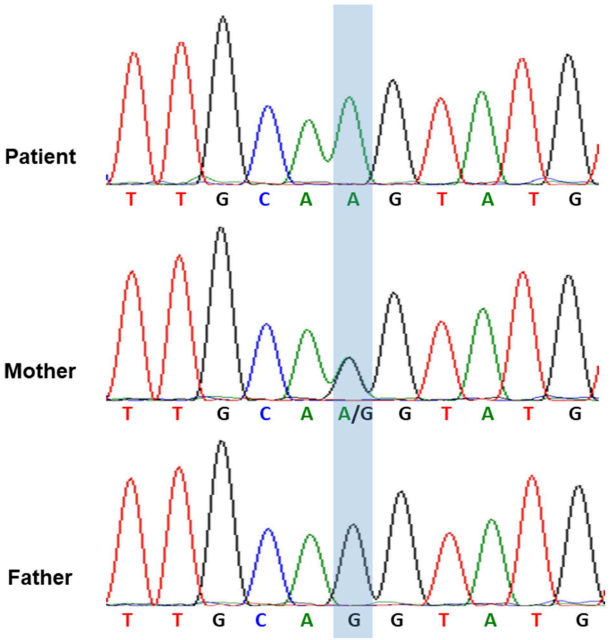

FIGURE 2 | The genetic analysis of BTK gene indicated that the hemizygous c.1632-1G>A mutation of *BTK* gene in the patient and the heterozygous c.1632-1G>A mutation of *BTK* gene in his mother.

A novel hemizygous c.1632-1G>A mutation in the *BTK* gene was discovered. This mutation was confirmed by Sanger sequencing.

The study was approved by the Institutional Review Board I&II of Taichung Veterans General Hospital, Taiwan (No. CF17231A), and written informed consent for publication of this case report was obtained from the parents of the patient.

Discussion

The genetic study of our patient revealed a novel hemizygous c.1632-1G>A mutation in the *BTK* gene on the X chromosome. This mutation affects the mRNA splice of the *BTK* gene, causing exon 17 to be skipped and leading to a frame-shift and premature termination codons (**Figures 3A,B**). Few reports have addressed HLH in patients with XLA (14, 15). Two male siblings with XLA and the *BTK* mutation were reported to exhibit HLH after adenovirus infection (14). One of the siblings died from the uncontrolled dissemination of adenovirus infections after receiving chemotherapy for HLH. The other sibling developed HLH later and survived because of the administration of Ig therapy instead of chemotherapy; he remained healthy under regular Ig replacement therapy (14). Another case report documented a 27-year-old man with undetectable circulatory B cells and selective IgM deficiency who exhibited HLH, which resolved after treatment with Ig replacement therapy (400 mg/kg), dexamethasone, and cyclosporine (15). The mechanism through which *BTK* mutation induces

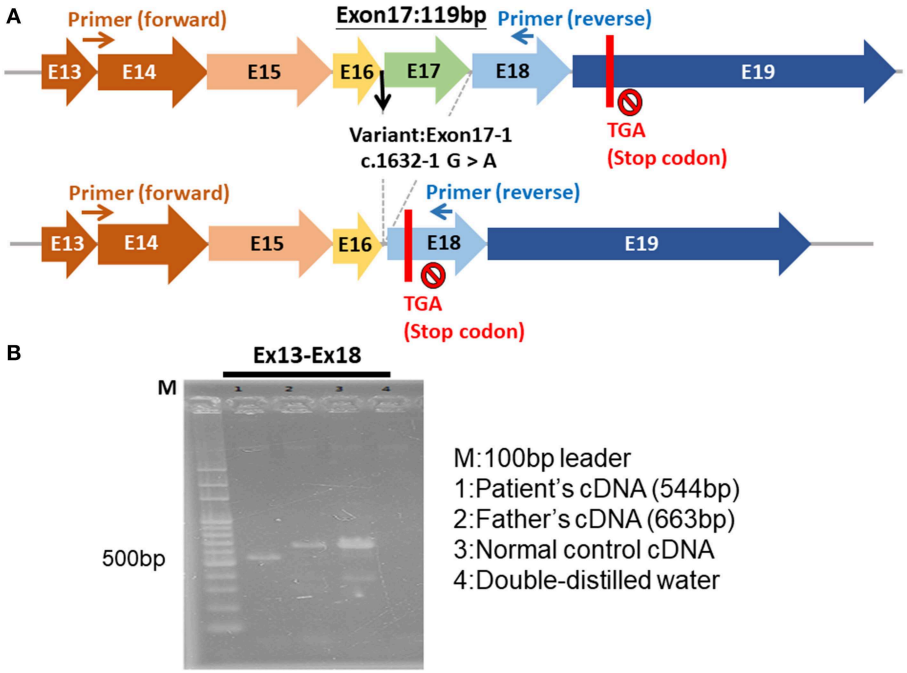

FIGURE 3 | The hemizygous c.1632-1G>A mutation of *BTK* gene. **(A)** The exon 17 was skipped, leading to a frame-shift and premature termination. **(B)** The electrophoresis of the cDNA of *BTK* gene of the patient (544 bp), patient's father (663 bp), normal control, and double-distilled water.

HLH remains unclear, but some studies have demonstrated that *BTK* participates in the activation of innate immunity through its involvement in the Toll-like receptors signaling pathway (16, 17).

The disease severity of XLA is influenced by the specific mutation in the *BTK* gene. Some *BTK* mutations can preserve some *BTK* enzyme activity, which is related to detectable circulating B cells, higher immunoglobulin levels, less severe clinical manifestations, and delayed diagnosis (18, 19). Thus, the transient elevated IgM in our patient can be partially explained. Other studies have reported the patients with atypical XLA with normal or near-normal levels of 1 or more certain immunoglobulin isotype(s) (19–21). These atypical XLA phenotypes were indistinguishable from other PIDs. Thus, the use of genetic analysis facilitates an accurate diagnosis.

The initial immunoglobulin data of our patient were obtained 1 month after the IVIg infusion (1 g/kg/day for 2 days) was administered to treat the second episode of HLH. He had undetectable plasma IgM and IgA levels, but the IgG level was within the normal range at that time. However, the IgG levels checked at the subsequent episodes of HLH showed a significant decrease before the commencement of IVIg treatment (1 g/kg/day for 2 days). The mutation in our patient was classified as a severe mutation because it occurred at the invariant sites of the splicing consensus sequence—the first and last 2 base pairs of the intron, which was consistent with the considerably decreased levels of all immunoglobulins in our patient. However, the significant increase in the IgM levels after a 6-month course of successive monthly Ig replacement (400 mg/Kg) suggested some mature B-cells were preserved in the patient (22). Moreover, it reminded us of the possibility of malignant change in a patient with immunodeficiency. IgM-secreting multiple myeloma and Waldenström's macroglobulinemia, both of which are very rare in adult patients and not seen in pediatric patients, were excluded because the plasma cells were <2% in the bone marrow biopsy survey. No evidence of lymphoma was discernible in the gallium scan. In the currently available publications, no IgM-secreting lymphoma, multiple myeloma, or Waldenström's macroglobulinemia has been reported in patients with XLA. The patient continued receiving monthly Ig infusions (400 mg/Kg) with the same dose and brand. IgM levels declined in the serial follow-ups and returned to the normal range 1 year later.

CONCLUSION

There is a broader range of clinical and immunological manifestations in XLA patients. Neither normal nor significantly increased immunoglobulin levels can exclude XLA. The analysis of the *BTK* gene helps to facilitate the accurate diagnosis. Additionally, HLH can be one of the severe complications of XLA. Physicians should be alert and consider Ig replacement therapy (400–600 mg/Kg every 3–4 weeks) to be the primary therapy for this condition.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the containing information affects the privacy of research participants. Requests to access the datasets should be directed to the corresponding author L-SF, linshienfu@yahoo.com.tw.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Institutional Review Board I&II of Taichung Veterans General Hospital, Taiwan, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Taichung Veteran General Hospital, Taiwan (No. CF17231A).

AUTHOR CONTRIBUTIONS

S-PH provided medical care to the patient, conceptualized the case report, collected data, and drafted the initial manuscript. S-FT, Y-FL, and H-YW participated in the genetic analysis, molecular biological experiment, and revised the manuscript in the genetic part. L-SF reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01953/full#supplementary-material

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Endocrine Disorders Are Prominent Clinical Features in Patients With Primary Antibody Deficiencies

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Background: Primary antibody deficiencies (PADs) and anterior pituitary dysfunction are both rare conditions. However, recent studies have remarkably reported the occurrence of anterior pituitary dysfunction in PAD patients.

Methods: In this cross-sectional, single-center study we evaluated the prevalence of endocrine disorders in adult PAD patients. Our study focused on common variable immunodeficiency (CVID), immunoglobulin G (IgG) subclass deficiency (IgGSD), and specific anti-polysaccharide antibody deficiency (SPAD). We assessed hormone levels, performed provocative tests and genetic testing in a subset of patients by direct sequencing of the nuclear factor kappa beta subunit 2 (*NFKB2*) gene and primary immunodeficiency (PID) gene panel testing by whole exome sequencing (WES).

Results: Our results demonstrated that one out of 24 IgGSD/SPAD patients had secondary hypothyroidism and three out of 9 men with IgGSD/SPAD had secondary hypogonadism. Premature ovarian failure was observed in four out of 9 women with CVID and primary testicular failure in one out of 15 men with CVID. In two out of 26 CVID patients we found partial adrenal insufficiency (AI) and in one out of 18 patients with IgGSD/SPAD secondary AI was found. Moreover, in one out of 23 patients with CVID and in two out of 17 patients with IgGSD/SPAD severe growth hormone deficiency (GHD) was found, while one patient with IgGSD/SPAD showed mild GHD. Combined endocrine disorders were detected in two women with CVID (either partial secondary AI or autoimmune thyroiditis with primary hypogonadism) and in three men with IgGSD/SPAD (two with either mild GHD or secondary hypothyroidism combined with secondary hypogonadism, and one man with secondary AI and severe GHD). Genetic testing in a subset of patients did not reveal pathogenic variants in *NFKB2* or other known PID-associated genes.

Conclusion: This is the first study to describe a high prevalence of both anterior pituitary and end-organ endocrine dysfunction in adult PAD patients. As these endocrine disorders may cause considerable health burden, assessment of endocrine axes should be considered in PAD patients.

Keywords: common variable immunodefiencies, endocrine disorders, immunodeficiency, endocrine dysfunction, hormones

INTRODUCTION

Primary antibody deficiencies (PADs) are the most common primary immunodeficiencies and are characterized by a B lymphocyte differentiation defect and an impaired production of antigen-specific antibodies resulting in increased risk of infections (1). PADs represent a heterogeneous spectrum of conditions, including common variable immunodeficiency (CVID), immunoglobulin G (IgG) subclass deficiency (IgGSD) and specific anti-polysaccharide antibody deficiency (SPAD).

CVID is a heterogeneous group of disorders primarily characterized by decreased serum IgG and IgA and/or IgM levels and impaired response to immunization (2, 3). CVID is clinically characterized by recurrent and severe infections, polyclonal lymphoproliferation, hematological malignancies, autoimmune diseases, and non-infectious granuloma formation in various organs (2, 3). IgGSD is defined by a reduction in one or more IgG subclasses (1–4), that may result in infectious diseases as well because of poor antibody responses (2, 3). SPAD is characterized by a reduced ability to produce antibodies against polysaccharide antigens (2, 3). Monogenetic defects responsible for CVID have been described in 2–10% of patients, while the genetic defects for IgGSD/SPAD remain unknown to date (3, 4).

Over the past years it has become clear that other, non-immunological comorbidities may occur in PAD patients. Although endocrine disorders are not regularly observed and reported in patients with PAD, recent studies suggest that anterior pituitary dysfunction is more common in patients with PAD than generally assumed.

Anterior pituitary dysfunction, with an estimated prevalence of 1:2,000, is the partial or complete defect in anterior pituitary hormone secretion and may result from pituitary or hypothalamic diseases (5). In 1991, Tovo et al. (6) reported the first case of a patient with CVID and the presence of an isolated adrenocorticotropic hormone (ACTH) deficiency, resulting in secondary adrenal insufficiency (AI) (dysfunction of the anterior pituitary). Since then, several other cases of CVID and secondary AI have been reported in literature (7–21). Quentien et al. (9) demonstrated isolated ACTH deficiency in four patients with CVID, including two siblings, and defined this disease association as a deficit in anterior pituitary function and variable immune deficiency (DAVID). Meanwhile, several cases have reported additional anterior pituitary and end-organ endocrine dysfunction in CVID patients. In particular, growth hormone deficiency (GHD) due to anterior pituitary dysfunction was repeatedly reported (2 patients with mild and 8 patients with severe GHD) (7, 9, 11, 15, 16, 21–24). Other distinct anterior pituitary defects and/or combined with endocrine end-organ dysfunctions were reported (7, 9, 11, 13, 15, 16, 19, 21, 22, 24, 25). The most important characterized reported cases are summarized in **Table 1** (6–11, 14, 15, 17–25).

Increased insights in causative genetic defects in CVID could potentially explain concomitant endocrine disorders. As an example, Chen et al. (10) reported germline variants near the C-terminal region in nuclear factor kappa beta subunit 2 (*NFKB2*) in CVID patients with secondary AI. Several other groups have also reported variants confined to the C-terminal region of the *NFKB2* gene that could cause combined endocrine- and immunodeficiencies and these are summarized in **Figure 1** (10, 12–16, 19–21, 26). It should be stressed that the NFKB signaling has a multitude of diverse functions within the immune system, and the hitherto published phenotypic observations of patients affected by *NFKB2* mutations were highly heterogenic (21).

To our knowledge no studies have attempted to systematically assess the prevalence of endocrine disorders in a cohort of PAD patients. The aim of our study is to investigate the prevalence of anterior pituitary and endocrine end-organ dysfunctions in adult patients with PADs from a tertiary referral center in the Netherlands.

METHODS

Patients and Ethics

In this single-center cross-sectional study, adult PAD patients were prospectively enrolled between May 2014 and November 2017. All patients ($n = 67$) were recruited from the outpatient clinics of the Department of Internal Medicine, Division of Clinical Immunology of the Erasmus University Medical Center, Rotterdam, the Netherlands. All CVID, IgGSD, and SPAD patients fulfilled the International Union of Immunological Societies (IUIS) expert committee diagnostic criteria (27). This study was carried out in accordance with the recommendations of the Medical Ethics Committee of the Erasmus University MC, Rotterdam with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Medical Ethics Committee of the Erasmus University MC, Rotterdam (MEC 2013-026, NL40331.078).

Hormonal Evaluation

Basal concentrations of anterior pituitary and endocrine end-organ hormones were measured including: ACTH, fasting cortisol, free thyroxine (FT4), thyroid-stimulating hormone (TSH), GH, insulin-like growth factor I (IGF-I), prolactin,

TABLE 1 | Described endocrine dysfunction in patients with CVID.

Patient no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Age at endocrine diagnosis (yr)	♀ **14** (6)	♀ **8** (7)	♂ **7** (8)	♀ **15** (9)	♀ **5** (9)	♂ **9** (9)	♂ **8** (9)	♀ **28** (10)	♀ **6** (10)	♂ **3** (10)	♂ **16** (10)	♀ **17** (11)	♀ **9** (12)	♂ **4** (13)	♀ **18** (14)
Familial relationship						**Full sibling of P7**	**Full sibling of P6**	**Mother of P13, 14**	**Daughter of P12, full sibling of P14**	**Son of P12, full sibling of P13**					
PADS / Endocrine dysfunctions	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**
Secondary AI															
Severe GHD															
Secondary hypothyroidism															
Mild GHD															
Secondary hypogonadism															
Primary hypogonadism															
Autoimmune thyroiditis															
Primary hypothyroidism															
Hyperprolactinemia															
Patient no.	**16**	**17**	**18**	**19-33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**
Age at endocrine diagnosis (yr)	♀ **8** (15)	♂ **7** (16)	♀ **5** (17)	**-** (18)	♀ **11** (19)	♂ **7** (20)	♂ **-** (21)	♂ **8** (21)	♂ **1** (21)	♀ (21)	♀ **-** (22)	♂ **-** (22)	♀ **10** (23)	♀ **24** (24)	♀ **24** (25)
Familial relationship						**Full sibling of P7**	**Full sibling of P6**	**Mother of P13, 14**	**Daughter of P12, full sibling of P14**	**Son of P12, full sibling of P13**					
PADS / Endocrine dysfunctions	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**	**CVID**
Secondary AI															
Severe GHD															
Secondary hypothyroidism															
Mild GHD															
Secondary hypogonadism															
Primary hypogonadism															
Autoimmune thyroiditis															
Primary hypothyroidism															
Hyperprolactinemia															

This table summarized anterior pituitary and end-organ endocrine dysfunction in CVID patients described in literature (6–25). AI, adrenal insufficiency; GHD, growth hormone deficiency; yr, years.

FIGURE 1 | This figure summarized reported germline variants confined to the C-terminal region of the NFKB2 gene in CVID patients with endocrine dysfunctions (10, 12–16, 19–21, 26). NFKB2, nuclear factor kappa beta subunit 2.

follicle-stimulating hormone (FSH), luteinizing hormone (LH), in women anti-mullerian hormone (AMH) and estradiol, and in men Inhibin B, sex hormone-binding globulin (SHBG) and testosterone. In patients who were on chronic corticosteroid therapy and who were not able to temporarily discontinue use of corticosteroids for at least 5 days, serum cortisol was not assessed. In all cases with abnormal TSH and/or free thyroxine levels, thyroid autoantibodies (i.e., anti-thyroid peroxidase; anti-TPO, anti-thyroglobulin; anti-Tg and anti-TSH-receptor; anti-TSH-R) were measured.

All blood samples were assessed at the routine Clinical Chemistry Laboratory (AKC) of Erasmus MC. In case patients were on intravenous immunoglobulin (IVIG) treatment, blood samples were collected prior to the IVIG infusion. All samples were collected during an apparent infection-free period, additional serum CRP was measured using Afinion™ Analyzer (Alere Ltd, Stockport, UK) to rule out acute endocrine disorders secondary to current inflammation.

Serum ACTH, cortisol, FSH, LH, prolactin, SHBG and TSH (Immulite 2000 XPi, Siemens AG), GH and IGF-I (IDS-iSYS; Immunodiagnostic Systems Limited, Boldon, UK), estradiol (Cobas e411, Roche Diagnostics GmbH) and free T4 (Vitros ECI, Ortho Clinical Diagnostics, Rochester, NY) were analyzed by automated immunoassays. IGF-I was interpreted according to the sex and age-dependent ranges as defined by Bidlingmaier et al. (28). Serum AMH (Beckman AMH gen II) and Inhibin B (Beckman Inhibin B gen II) were manually determined by ELISA. Testosterone was determined by liquid chromatography-tandem mass spectrometry (PerkinElmer CHS™ MSMS Steroids Kit on a Waters Xevo TQS). Furthermore, serum anti-TPO and anti-TSH-R were measured with and in-house ELISA and anti-Tg was assessed using the ImmunoCAP method (Phadia 250, Uppsala, Sweden).

Provocative Testing

Provocative testing was performed at our inpatient clinic on a separate occasion when AI and/or GHD was suspected or when patients had another anterior pituitary deficiency. AI was suspected in patients with a fasting basal serum cortisol <266 nmol/L. In patients with basal serum cortisol 266–550 nmol/L, AI is not excluded. Therefore, basal serum cortisol was repeated in these cases and when a second result was still below 550 nmol/L, based on our assay-specific normative data, a provocative test was performed. GHD is suspected in patients when IGF-I levels were below −1 SDs according to Bidlingmaier et al. (28).

Insulin tolerance test (ITT) was used for the assessment of AI or GHD. The ITT was carried out before breakfast after overnight fasting. Venous blood samples were drawn for assessment of cortisol, ACTH, GH, and glucose before (−15 min), and at 0, 20, 30, 40, 60, and 90 min after administration of i.v. insulin. In patients in whom an ITT was contraindicated (i.e., history of coronary artery disease, seizures or stroke; age > 65 is a relative contraindication), an overnight metyrapone test was performed. The test starts at day 1 at 8 a.m. with oral administration of 750 mg of metyrapone, followed by 6 doses additional administration every 4 h. Serum cortisol and 11-deoxycortisol were measured as described above at 8 a.m. at day 2. Growth hormone releasing hormone (GHRH)-arginine test was only performed in patients with isolated decreased IGF-I levels or when ITT results remained inconclusive. The GHRH-arginine test starts by administration of i.v. GHRH (1 μg GHRH/kg bodyweight; BW), followed by a 30 min continuous infusion of 0.5 g arginine/kg BW followed by measurement of GH at −15, 0, 5, 10, 15, 20, 30, 45, 60, 75 and 90 min. In patients who were not able to temporarily discontinue use of corticosteroids for at least 5 days, provocative testing was not performed. The biochemical tests and diagnostic methods were performed in a specialized accredited laboratory for endocrine diseases and confirmed by a senior neuro-endocrinologist (S.N.).

Neuroradiological Imaging

Magnetic resonance imaging (MRI) of the pituitary was performed according to standard operation procedures of the department of neuroradiology of the Erasmus MC.

Genetic Analysis

Direct Sanger sequencing of the two C-terminal coding exons of the *NFKB2* gene was performed in a selected group of PAD patients with endocrine dysfunction ($n = 16$). We focused on the *NFKB2* gene expression based on previous reports (10, 12–16, 19–21, 26) and its known function in both the immune and endocrine systems. DNA was extracted from peripheral blood samples using standard protocols. *NFKB2* exon 22 and 23 were PCR-amplified with TaqGold™ (Life Technologies) followed by direct sequencing on an ABI Prism 3130 XL fluorescent sequencer (Applied Biosystems, The Netherlands). Sequences were analyzed with CLC DNA workbench software (CLCBio, Aarhus, Denmark) and compared to the NCBI reference sequence (NG_033874).

Additionally, in eight patients PID gene panel testing comprising over 250 PID-associated genes [range 274–367; based on the IUIS classifications 2015 (29) and 2017 (3) was performed using whole exome sequencing (WES). DNA was enriched for the exome using the Agilent Sureselect Clinical Research Exome V2 Capture Enrichment kit (Agilent Technologies) and paired-end sequenced on the Illumina Hiseq platform (GenomeScan, Leiden, the Netherlands). Using our sequencing protocols, the average coverage of the exome is ∼50X. Reads were mapped to the genome with the BWA-MEM algorithm (http://bio-bwa.sourceforge.net/) and variant calling was performed by the Genome Analysis Toolkit HaplotypeCaller (http://www.broadinstitute.org/gatk/). Detected variants in the PID-associated genes were filtered and annotated with the Cartagenia software package and classified with Alamut Visual. Detailed information for each panel is listed in **Table S1**.

Statistical Analysis

Statistical analyses were performed using SPSS software (version 21 for Windows; SPSS Inc., Chicago, Illinois). Descriptive statistics were used to summarize patient characteristics. The non-parametric unpaired two-samples Wilcoxon test, the Pearson chi-square tests or the Fisher's exact test were used to determine the significance of difference between CVID and IgGSD/SPAD patients. We considered *P*-values of < 0.05 to be statistically significant, *P*-values were not corrected for multiple comparisons.

RESULTS

Patient Characteristics

The baseline characteristics of CVID and IgGSD/SPAD patients groups are summarized in **Table 2**. A total of 67 patients were included in our study and are categorized according to the type of PAD; 43 patients with CVID, 16 patients with IgGSD and 8 patients with SPAD. There is no familial relationship between the patients. Thirty-nine patients received IVIG treatment at doses between 20 and 80 g/4 weeks and 22 patients were on subcutaneous Ig (ScIg) treatment at a dose of 19–53 g/4 weeks. Six patients were not treated with Ig replacement therapy. A significant difference in the number of patients receiving Ig replacement therapy vs. patients receiving no Ig replacement therapy was present between the CVID and IgGSD/SPAD group (**Table 2**). In all patients the serum CRP levels were within normal range according to reference values from the AKC of Erasmus MC.

TABLE 2 | Baseline characteristics of included patient groups.

Demographic variable	CVID	IgGSD/SPAD	*p*-value
DIAGNOSIS CVID	43 (64.2)		
IgGSD		16 (23.9)	
SPAD		8 (11.9)	
Mean age at evaluation, yr	47 (17.0)	53 (14.5)	0.099
Male	15 (34.9)	9 (37.5)	0.830
Receive Ig substitution	42 (97.7)	19 (79.2)	0.020

Data are mean (SD) or n (%). Ig, immunoglobulin; yr, years.

Endocrine Disorders

All anterior pituitary and end-organ endocrine dysfunction found in our cohort are summarized in **Table 3**.

Hypothalamic-Pituitary-Gonadal (HPG) Axis

In three out of 9 men with IgGSD/SPAD secondary hypogonadism due to anterior pituitary dysfunction was diagnosed. Male primary gonadal dysfunction was found in one out of 15 men with CVID who presented with primary testicular failure (i.e., low testosterone and high LH and FSH levels) and was treated with testosterone replacement therapy. There were no clinical indications for Klinefelter syndrome or idiopathic granulomatous orchitis, and therefore, we did not obtain karyotype or evaluate for antisperm antibodies.

Primary gonadal dysfunction (i.e., primary hypogonadism) in women was identified in four out of 9 CVID patients (aged ≤40 years) that had premature ovarian failure based on low AMH, low estrogen and high LH and FSH levels. Two of these patients were treated with combined oral contraceptive pill. We did not evaluate for adrenal antibodies or thyroid antibodies.

Hypothalamic-Pituitary-Thyroid (HPT) Axis

Hypothyroidism with normal TSH levels in one man out of 24 IgGSD/SPAD patients was considered to be due to secondary hypothyroidism (i.e., a dysfunction of the anterior pituitary gland) and was treated with thyroxine replacement therapy.

In four women thyroid dysfunction was found; one woman out of 43 CVID patients demonstrated subclinical hypothyroidism, defined as elevated TSH with normal free thyroxine levels, whereas one of the 24 patients with IgGSD/SPAD (female) had subclinical hypothyroidism including the presence of thyroid antibodies. Autoimmune thyroiditis was identified in two women with CVID; one had Graves' disease and the other had granulomatous thyroiditis without the presence of thyroid antibodies. The granulomatous thyroiditis was detected with somatostatin receptor scintigraphy with ^{111}In-pentetreotide (OctreoScan) and confirmed by fine-needle aspiration biopsy of the thyroid gland. However, we cannot rule

out that the hypothalamus nor the pituitary gland was affected by granulomatous disease. This area is physiologically positive on OctreoScan. However, patients with granulomatous hypophysitis may benefit from high-dose corticosteroid therapy, which was initiated in this patient when granulomatous thyroiditis was diagnosed.

Hypothalamic-Pituitary-Adrenal (HPA) and Somatotropic Axis

Provocative testing was performed when AI or GHD was suspected. ITT was performed in 34 patients, metyrapone test in 9 patients and GHRH-arginine test in 8 patients.

In two out of 26 CVID patients partial secondary AI was observed, where cortisol levels remained below 400–550 nmol/L during ITT. In one of them hydrocortisone replacement therapy was initiated during follow-up due to persistent low cortisol levels. The other patient did not present with symptoms or signs of hypocortisolism but cortisol levels remained suboptimal during follow-up. Therefore, we provided stress instructions and added to the medical record that hydrocortisone replacement therapy was necessary in case of a medical emergency. One out of 18 patients with IgGSD/SPAD secondary AI was observed during ITT and was treated with hydrocortisone replacement therapy.

Severe GHD was observed in one out of 23 patients with CVID and in two out of 17 patients with IgGSD/SPAD. All three patients were treated with GH replacement therapy. Moreover, in one patient in the IgGSD/SPAD group mild GHD was observed.

Combined Endocrine Disorders

As was already mentioned above, some patients showed multiple endocrine disorders. Combined anterior pituitary dysfunction was detected in three men with IgGSD/SPAD; one had severe GHD with secondary AI, the second mild GHD and secondary hypogonadism, while the last patient had secondary hypothyroidism and secondary hypogonadism.

In one woman with CVID we detected partial secondary AI and primary hypogonadism, which is a combination of anterior pituitary dysfunction and end-organ dysfunction. Combined endocrine end-organ dysfunction was found in one woman with CVID who presented with granulomatous thyroiditis as well as primary hypogonadism.

MRI scans of the pituitary were performed in the three patients with severe GHD and showed normal pituitary anatomy. It should be noted that pituitary anatomy visualized by MRI is not performed in other patients.

Genetic Analysis

To evaluate the involvement of *NFKB2* in PAD patients with endocrine dysfunctions, these patients (n = 16) were analyzed by sequencing *NFKB2* exon 22 and 23 (**Table 3**). No pathogenic variant in the C-terminal region of *NFKB2* was detected in any of these patients.

Additionally, 8 out of the 67 PAD patients were investigated for pathogenic variants in PID-associated genes. In one patient (**Table 3**; no. 1) a heterozygous nuclear factor kappa beta inhibitor alpha (*NFKBIA*)-variant (NM_020529.2(*NFKBIA*):c.554C>T, p.(Thr185Met)) with

TABLE 3 | Described endocrine dysfunction in patients with CVID and IgGSD/SPAD.

Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Age at endocrine diagnosis (yr)	♂ 42	♂ 45	♂ 51	♂ 39	♂ 68	♀ 39	♀ 41	♂ 77	♀ 42	♂ 65	♀ 34	♀ 19	♀ 41	♀ 81	♀ 50	♀ 25
PADS / Endocrine dysfunctions	IgGSD/SPAD	IgGSD/SPAD	IgGSD/SPAD	CVID	IgGSD/SPAD	CVID	CVID	IgGSD/SPAD	CVID	CVID	CVID	CVID	IgGSD/SPAD	CVID	CVID	IgGSD/SPAD
Secondary AI																
Partial secondary AI																
Severe GHD																
Mild GHD																
Secondary hypothyroidism																
Autoimmune thyroiditis																
Subclinical hypothyroidism																
Secondary hypogonadism																
Primary hypogonadism																

This table summarized anterior pituitary and end-organ endocrine dysfunction found in our cohort. AI, adrenal insufficiency; GHD, growth hormone deficiency; yr, years.

unknown significance was detected. This variant was not identified in the unaffected father; the mother was unavailable for testing. However, the minor allele frequency in control populations (Genome Aggregation Database v2.1.1; http://gnomad.broadinstitute.org/) was 0,026% (72 out of 282058 alleles) and functional assays in patients cells did not show impairment of *NFKB*-activation and cytokine production (data not shown). No other pathogenic variants or candidates were detected in PID-associated genes in the eight PAD patients.

DISCUSSION

To our knowledge, this is the first cross-sectional study on the prevalence of endocrine disorders in adult PAD patients. We not only reconfirmed previous findings demonstrating the presence of endocrine disorders of anterior pituitary origin in PAD patients, but we also found end-organ endocrine dysfunction with or without anterior pituitary hormone dysfunction. Moreover, the prevalence of endocrine disorders was unexpectedly high in our patients with CVID, IgGSD, and SPAD.

Our results confirm previous findings that severe GHD is present in CVID patients (7, 11, 15, 21–23). We also observed premature ovarian failure due to end-organ dysfunction in four CVID patients, which was previously described in one case as well (24).

In this study we observed for the first time co-existence of partial secondary AI due to anterior pituitary dysfunction in association with CVID. Moreover, it is also the first time we observed primary testicular failure, subclinical hypothyroidism, granulomatous thyroiditis and Graves' disease due to end-organ dysfunction in CVID patients. Noteworthy is that Graves' disease is a common autoimmune thyroid disease, but since patients with CVID exhibit multiple autoimmune phenomena, we cannot rule out Graves' disease as a comorbidity in CVID. Also, granulomatous complications are a well-known comorbidity of CVID, particularly in the lungs, but these granulomas can be present in all organs, which fits with the presence of granulomatous thyroiditis in one of our CVID patients.

We show for the first time the occurrence of endocrine disorders in adult patients with IgGSD/SPAD. The endocrine disorders of anterior pituitary origin that we observed in the IgGSD/SPAD group were secondary hypogonadism, secondary hypothyroidism, secondary AI and mild and severe GHD. In the latter, MRI scans showed normal pituitary anatomy. Therefore, structural abnormalities (such as an expanding intrasellar mass) or hypophysitis due to autoimmunity or auto-inflammatory conditions are unlikely as causes for GH deficiency. A common genetic cause may explain concomitant GH deficiency in PAD. Moreover, chronic illness is also associated with suppressed GH levels (30) and may account for the high incidence of GH deficiency in PAD patients. Besides, we observed subclinical hypothyroidism due to end-organ dysfunction in the IgGSD/SPAD patients. Although, anterior pituitary hormone deficiencies occurred even more in the IgGSD/SPAD group than in the CVID group, these dysfunctions are not previously described in literature.

PADs and disorders caused by deficiencies in anterior pituitary function are both rare conditions. The high prevalence of endocrine disorders in PAD patients as described in our study may suggests that PADs and anterior pituitary dysfunction are not two distinct phenomena in these patients, but could be both the result of a single pathologic condition.

Previous studies have shown that variant(s) in *NFKB2* (10, 12–16, 19–21, 26) could be considered as underlying genetic defects resulting in both B cell immunodeficiency and endocrine dysfunction. *NFKB2* encodes the full-length p100 protein and serves as central player of the non-canonical *NFKB* signaling pathway, which has a critical role in pituitary development, particularly in differentiation of ACTH-producing corticotroph cells. Interestingly, all of the *NFKB2* variants reported are near the C-terminus of the protein-coding region of *NFKB2*, a region required for the correct processing of the primary translation product (31, 32). It is important to emphasize that the *NFKB2* mutations described so far showed heterogenic clinical expressivity (21) and are associated with a variable penetrance (16), which makes it difficult to predict the phenotype based on the genetic alteration. However, in all our 16 patients with endocrine dysfunctions no pathogenic variants in the C-terminal region of *NFKB2* was detected. Except for the *NFKBIA*-variant of unknown significance in patient no. 1, we did not detect other pathogenic variants in PID-associated genes in eight PAD patients.

We did not identify pathological variation in the coding sequence of the *NFKB2* gene tested, but we believe that the observations described support the existence of a disease association possibly related to a common genetic link. Absence of identification of sequence abnormalities in the open reading frame of the *NFKB2* gene tested might be due to the fact that our study was limited to the coding exons of the gene. Alternatively, it may be explained by the involvement of a number of other currently identified or unknown genes. To elucidate this issue, the possible common molecular cause for combined endocrine- and immunodeficiencies is presently being investigated in our cohort.

Endocrine disorders are not only found in PADs, but are also common features in patients with other primary immunodeficiency diseases (PIDs), such as type 1 diabetes mellitus and thyroiditis in *STAT1* gain-of function (GOF) variant (3), type 1 diabetes mellitus and short statue in *STAT3* GOF variant (33), hypoparathyroidism in DiGeorge syndrome (3) and hypo(para)thyroidism and AI in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (3). Future genetic studies may reveal more common genetic pathways involved in immunodeficiency and endocrine dysfunction.

Our report has several limitations. Firstly, we excluded patients who were not able to temporarily discontinue their use of corticosteroids, which interferes with provocative testing. Therefore, the number of patients in which secondary AI was found could be underestimated. Secondly, in our cohort almost

all patients were on Ig replacement therapy during the study. The way in which Ig replacement therapy exerts immunomodulatory effects remains unclear, with many pathways, probably mutually non-exclusive, in the innate and adaptive immune systems being potentially targeted. Finally, we focused on the *NFKB2* gene expression only in a selected group of PAD patients with distinctive endocrine dysfunction. Moreover, PID gene panel testing by WES was only performed in eight patients for diagnostic purposes. From these patients, results were included in the current study.

In this study we have observed a remarkably high prevalence of pituitary and end-organ dysfunctions in adult patients with CVID and we expand the phenotype by including IgGSD and SPAD. However, it is not known until now how the immunological and molecular defects in PADs contribute to the development of endocrine disorders. Further genetic studies in PADs may provide new insights into molecular and/or autoimmune mechanisms that could result in these endocrine comorbidities. Endocrine comorbidities in PAD may result in a considerable health burden, we recommend that clinicians consider endocrine disorders in PAD patients and screen for endocrine dysfunction when appropriate.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Medical Ethics Committee of the Erasmus University MC, Rotterdam with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Medical Ethics Committee of the Erasmus University MC, Rotterdam (MEC 2013-026, NL40331.078).

AUTHOR CONTRIBUTIONS

EC, A-JL, PH, SN, and VD contributed to the study design. EC, PC, and ME collected and provided primary patient data and EC, A-JL, PH, SN, and VD analyzed the clinical results. EC, BB, SS, IH, and PS performed analysis and interpretation of data from genetic testing. EC and VD drafted the manuscript. All other authors were involved in critical revision of the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02079/full#supplementary-material

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Current Understanding and Recent Developments in Common Variable Immunodeficiency Associated Autoimmunity

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Common variable immunodeficiency (CVID) is the most prevalent symptomatic primary immunodeficiency and comprises a group of disorders with similar antibody deficiency but a myriad of different etiologies, most of which remain undefined. The variable aspect of CVID refers to the approximately half of patients who develop non-infectious complications in addition to heightened susceptibility to infection. The pathogenesis of these complications is poorly understood and somewhat counterintuitive because these patients that are defined by their immune futility simultaneously have elevated propensity for autoimmune disease. There are numerous aspects of immune dysregulation associated with autoimmunity in CVID that have only begun to be studied. These findings include elevations of T helper type 1 and follicular helper T cells and B cells expressing low levels of CD21 as well as reciprocal decreases in regulatory T cells and isotype-switched memory B cells. Recently, advances in genomics have furthered our understanding of the fundamental biology underlying autoimmunity in CVID and led to precision therapeutic approaches. However, these genetic etiologies are also associated with clinical heterogeneity and incomplete penetrance, highlighting the fact that continued research efforts remain necessary to optimize treatment. Additional factors, such as commensal microbial dysbiosis, remain to be better elucidated. Thus, while recent advances in our understanding of CVID-associated autoimmunity have been exciting and substantial, these current scientific advances must now serve as building blocks for the next stages of discovery.

Keywords: primary immunodeficieny, common variable immunodeficiency, autoimmunity, cytopenia, genetics, microbiome, precision therapy

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INTRODUCTION

The diagnosis common variable immunodeficiency (CVID) is used to denote a group of disorders that, together, account for more than 50% of symptomatic primary immune deficiencies (PID) (1) with an estimated incidence of 1:50,000–1:25,000 (2). The term common variable immunodeficiency was originally used to describe patients with a primary antibody deficiency who did not meet criteria for the more well-defined PIDs such as Burton's agammaglobulinemia (3). We have come to appreciate that CVID is best understood to constitute a group of

hypogammaglobulinemic disorders with heterogeneous phenotypic presentations, rather than a single entity (4). The application of genomics has only furthered the evidence of heterogeneity within CVID.

Diagnostic criteria for CVID have evolved since it was first made a diagnosis of exclusion in 1971 by the World Health Organization. The International Consensus Document on CVID put forward in 2016 agreed on a definition requiring IgG levels two standard deviations below the age-appropriate reference as well as either low IgA or IgM levels, and poor antibody response to vaccination in an individual that is at least 4 years old with no secondary cause of hypogammaglobulinemia (1). The European Society of Immune Deficiencies (ESID) diagnostic criteria differ slightly from these criteria in that they require the presence of symptoms such as infections or autoimmune manifestations, in addition to the laboratory abnormalities cited above, to make the diagnosis of CVID (5). Notably, non-infectious presentations are an under-recognized feature of CVID and often the predominant clinical presentation, resulting in diagnostic delays of several years in many cases (6). While historically the clinical presentation of CVID was focused on the susceptibility to infections, the decision to include autoimmune and inflammatory conditions as primary clinical presentations reflects the heterogeneity of CVID and highlights the importance of recognizing these non-infectious entities as a feature of this immune deficiency.

Immunoglobulin replacement therapy is the standard of care for CVID. Since the widespread adoption of this treatment, mortality of patients with CVID has decreased from 30% in the early 1990s (7) to 15% in the early 2000s (8), in a cohort of 240 patients in the United Kingdom and 334 patients from the ESID registry, respectively, all followed for approximately two decades. The improved survival in CVID patients has been attributed to the reduction of infectious complications thanks to the widespread use of immunoglobulin replacement and improved anti-microbial therapies (9–12). While overall survival has improved, patients with CVID continue to have reduced survival compared to age-matched controls (13). When comparing mortality within CVID patients, subjects with at least one non-infectious complication had significantly higher mortality compared to patients with only infectious complications (13, 14). These non-infectious complications including autoimmune, gastrointestinal, pulmonary, lymphoproliferative, and malignant complications (1), are not ameliorated by immunoglobulin replacement therapy alone. The clear necessity to address non-infectious complications of CVID has led to attempts at categorizing these heterogeneous disorders into distinct phenotypes, with the hopes of elucidating endotypes. The ultimate goal of such stratification of CVID is to identify targeted treatments that will improve outcomes in CVID patients with non-infectious complications (14–16).

The first attempt at phenotyping CVID patients was done by Chapel et al. (14) by categorizing the various associated complications and looking at the independence of one from the other. They identified five distinct phenotypes: no complications, autoimmunity, polyclonal lymphocytic infiltration, enteropathy, and lymphoid malignancy (14). Studies that followed have confirmed categorization of CVID based upon the presence of complications, with certain features like autoimmunity, lymphocytic interstitial lung disease, and lymphoid hyperplasia typically occurring together (6, 17). Assuming that CVID endotypes present within these phenotypic clusters, the pathogenesis and genetic mechanisms underlying the disease may be related. Autoimmunity and immune deficiency have been shown to have genetic overlap and occur together beyond CVID (18–20), suggesting a common pathophysiologic mechanism underlying both forms of immune dysregulation. In this review, we focus on one aspect of immune dysregulation, the autoimmune manifestations of CVID, providing an overview of these complications as well as an update on research and treatment advances.

OVERVIEW OF AUTOIMMUNE DISEASE IN COMMON VARIABLE IMMUNODEFICIENCY

The initial clustering of CVID patients with autoimmunity included organ specific autoimmune disease (e.g., Grave's thyroiditis, insulin dependent diabetes mellitus), systemic autoimmune disease (e.g., rheumatoid arthritis, systemic lupus erythematosus), and autoimmune cytopenias (e.g., immune thrombocytopenia, autoimmune hemolytic anemia) (14). Further analysis on two other cohorts showed that within the autoimmune cluster, only autoimmune cytopenias had decreased survival and that organ-specific and systemic autoimmune disease showed no association with cytopenias or the other clinical phenotypes (16). This led to a revision of the clinical phenotypes with more emphasis being placed on autoimmune cytopenias as opposed to autoimmunity in general (16). Unbiased network clustering in a separate CVID cohort yielded similar phenotypes, with systemic and organ-specific autoimmune diseases clustering separately from autoimmune cytopenias (21). While this distinction likely carries implications regarding underlying pathophysiology, many studies continue to combine autoimmune cytopenias with other autoimmunity as they compare CVID patients. For this reason, we will make distinctions between autoimmune cytopenias and organ specific or systemic autoimmunity in this review.

Autoimmune diseases are one of the most common non-infectious complications, occurring in ∼20–30% of patients with CVID, with autoimmune cytopenias being the most common (9, 22, 23). In a European cohort of 2700 CVID patients taken from the ESID registry, autoimmune cytopenias were found to be 700 times more prevalent in CVID patients compared to the general population (9). Among autoimmune cytopenias, autoimmune thrombocytopenia and autoimmune hemolytic anemia occur most frequently, either separately or concurrently as Evan's syndrome. Autoimmune neutropenia also occurs in CVID, although more rarely than thrombocytopenia or anemia (9, 24). Importantly, the diagnosis of cytopenia precedes that of CVID by several years in up to 60% of patients (22).

Autoimmune cytopenias are often associated with other non-infectious complications in CVID. Compared with other

CVID patients, those with ILD are more likely to have had autoimmune cytopenias (6, 25). Conversely, CVID patients with autoimmune cytopenias had a higher frequency of CVID-associated non-infectious complications, including granulomatous and lymphoproliferative disease, as well as organ-specific autoimmune disease, but interestingly, not systemic autoimmunity (26). In a recent study of a 295 patient CVID cohort in the Czech Republic, immune thrombocytopenic purpura (ITP) was identified as a risk factor for malignancy, with over a 3-fold increase compared to those without ITP (27). Splenomegaly was also more common in patients with autoimmune cytopenias and was found to share some immunophenotypic characteristics, but the pathophysiology of this link is still not clearly understood (28).

Though autoimmune cytopenias are highly associated with CVID, other forms of autoimmunity have also been frequently reported. In a recent study of 870 CVID patients from the USIDNET registry, 5% were found to have rheumatologic disease, which accounted for 40% of the autoimmune complications of this cohort. Although the male-to-female ratio was almost equal for overall autoimmune complications, there was a clear female predominance for the rheumatologic manifestations (24). Several other studies have shown a similar female predominance for systemic autoimmune complications in CVID (29, 30). The most common rheumatologic manifestation reported in CVID is inflammatory arthritis (juvenile and adult), occurring in ∼3% of patients (13, 24, 31, 32). Other rheumatologic manifestations include systemic lupus erythematosus, Sjogren's disease, Behcet's disease, and psoriasis (24, 32). Among organ specific autoimmune manifestations, hypothyroidism was the most prevalent at 3.5%, followed by alopecia areata and vitiligo at 2.7%, and type 1 diabetes at 1.6% in an ESID registry of 2700 CVID patients (9). Autoimmunity may also underlie gastrointestinal complications of CVID, including inflammatory bowel disease, autoimmune enteropathy, and autoimmune gastritis (33).

IMMUNOPHENOTYPIC MARKERS ASSOCIATED WITH AUTOIMMUNITY IN CVID AND IMPLICATIONS ON PATHOGENESIS

T Cell Dysregulation in CVID Autoimmune Disease

While PIDs in general are associated with a higher risk of autoimmune manifestations, when comparing across all PIDs, the greatest risk of autoimmune cytopenia and rheumatologic disease was seen in two specific subsets of PID: CVID and PID with T-cell deficiencies (34). This finding highlights the importance of T cell dysfunction in CVID autoimmunity and demonstrates that pathogenesis of this immune disorder extends beyond defects of B cells exclusively. Indeed, CVID patients with autoimmunity have been found to have lower total T cells compared to those without autoimmunity (30). Such T cell deficiency is not necessarily profound, as a CD4+ T cell count <200 or significantly impaired lymphocyte proliferation may result in categorization as a combined immunodeficiency rather than CVID (35). When Chapel et al. classified CVID patients into specific phenotypes, they found that low proportions of CD8+ T cells were predictive of autoimmunity; in fact, in this cohort, each additional 10% increase in CD8+ T cells reduced the odds of autoimmunity by 18% (14). Later studies found that autoimmunity in CVID is associated with lower naïve CD8+ T cells specifically, with terminally differentiated CD8+ T cells actually increased, suggesting a hyperactivated T cell phenotype as the defining feature (36). While several studies have shown reduced CD4+ cells in CVID (29, 37), those with autoimmune cytopenias and organ specific autoimmunity had the most significantly reduced CD4+ T cells (36, 38, 39). Within CD4+ T cells, autoimmunity in CVID was particularly associated with reduced number of regulatory T cells (T_R) compared to CVID patients without autoimmunity (39, 40). Expression of the canonical T_R transcription factor forkhead box P3 (FOXP3) was reduced in CVID patients with autoimmunity compared to those without (39), and the suppressive activity of T_R cells in CVID with autoimmunity was also reduced, with the degree of dysfunction correlating with the extent of FOXP3 downregulation (41). Other findings in CVID patients with autoimmunity include lower CCR7+ T cells, also considered to be key mediators in immune tolerance (42). Overall, T cell-mediated processes that help promote immune tolerance appear to break down in a subset of CVID patients, contributing to the development of autoimmunity.

In addition to loss of naïve and regulatory T cells, an increase in T helper type 1 (T_{H1}) and T follicular helper (T_{FH}) CD4+ T cells have been described in association with autoimmunity in CVID. T_{FH} cells provide help to activate and diversify B cell responses within secondary and tertiary lymphoid tissues (43). T_{FH} are elevated in CVID patients with autoimmunity, particularly those producing type 1 cytokines or otherwise known as T_{FH1} (44). While T_{FH} provide most of their function within germinal centers, it is notable that their increase is associated with germinal center enlargement and disorganization in CVID patients with autoimmunity (45). This increased T_{FH} development has been linked with greater IgA deficiency and resultant endotoxemia, presumably due to bacterial translocation from mucosal surfaces in the absence of IgA (46). Expansion of T_{H17} cells has also been associated with autoimmunity in patients with CVID (47, 48). Thus, it is clear that T cell dysregulation, particularly loss of regulatory subsets with concurrent increase in proinflammatory lymphocytes, is a fundamental feature of CVID patients with autoimmunity. Continued efforts toward improved understanding of this form of immune dysregulation will be vital to improving treatment of CVID associated autoimmune disease.

B Cell Dysfunction in CVID Autoimmune Disease

Low frequency of T_R cells in CVID patients with autoimmunity is associated with expansion of a particular B cell type, $CD21^{low}$ B cell (37), linking T and B cell pathology in CVID. Early studies have shown that reduced switched memory B cell (CD19+CD27+IgD−) percentage correlates more strongly with

autoimmunity in patients with CVID compared to serum IgG levels (49). Indeed, patients with reduced numbers of switched memory B cells (≤0.55% of B-cells) had greater than a 3-fold increase in their risk of autoimmune cytopenias and systemic autoimmune disease (50). Subsequent studies found increased proportions of $CD21^{low}$ B cells in patients with CVID (37, 51, 52), with clustering of these low levels in patients with autoimmunity (28, 53). Interestingly, non-CVID patients with autoimmune disease have also been found to have expansion of $CD21^{low}$ B cells (54). These cells were found to have preferential homing to peripheral tissues such as the synovial fluid of rheumatoid arthritis patients and the bronchoalveolar space of CVID patients (55). Further analysis found that $CD21^{low}$ B cells from both rheumatoid arthritis and CVID patients expressed germline autoreactive antibodies which recognized nuclear and cytoplasmic structures (56). Authors concluded that $CD21^{low}$ B cells are a "distinct, polyclonal, pre-activated, partially autoreactive, functionally attenuated B cell population with preferential enrichment in peripheral tissues" thus offering another possible mechanism of autoimmunity in patients with CVID (55).

Notably, $CD21^{low}$ B cells were found to produce significantly more IgM than naïve B cells after stimulation with CD40L, IL-2, and IL-10 (55). Along these lines, CVID patients with autoimmunity have been found to have higher levels of IgM compared with non-autoimmune phenotypes (31, 57). High levels of IgM have also been associated with autoimmunity in other PIDs, such as Wiskott-Aldrich syndrome (58). While this may be a marker for increased risk of autoimmune disease, and may be related to the aforementioned $CD21^{low}$ B cells, there may be a pathogenic role for IgM autoantibodies, as IgM may underlie the autoimmune cytopenias that are the predominant autoimmune manifestation of CVID (57).

B cell receptor diversity is diminished in CVID patients with autoimmunity (59). As a consequence of this diminished B cell repertoire, the presence of certain B cell receptors with autoreactive proclivity, such as those that express the VH4-34 heavy chain, may be more prominent in CVID patients as has been demonstrated in other forms of PID associated with autoimmunity (60, 61). Thus, B cell developmental defects that impair immunity may also contribute to the propensity for autoimmunity in some CVID patients.

B cell-activating factor (BAFF), a cytokine that promotes both maturation and survival of B cells, has long been linked with autoimmunity when its levels are elevated (62–64). BAFF also stimulates T cells via the BAFF receptor (BAFF-R) and skews the inflammatory response by augmenting T_{H1} cytokine production (65). Though BAFF levels were found to be elevated in CVID, an association with autoimmunity was not found (66). One possible explanation for this discrepancy is that the sample size of this cohort did not provide enough power to reach significance (only 17 of 77 patients had autoimmunity). In a separate study, BAFF levels were elevated in CVID patients with active interstitial lung disease, an inflammatory pulmonary disease linked with autoimmune cytopenias (17, 25). On the other hand, BAFF levels were not elevated in those with quiescent stable disease, suggesting that increases of this cytokine might be closely tied to disease activity (67) thus offering another possible explanation for the lack of association in the aforementioned cohort. While shedding of BAFF receptors has been postulated to regulate BAFF-driven inflammation, there was no relationship of serum levels of the BAFF receptor B cell maturation antigen (BCMA) and the development of autoimmunity in CVID (68, 69). Yet, elevated BAFF levels have been shown to inhibit negative selection of autoreactive B cells, in CVID autoimmunity as in other diseases, which apparently contributes to the increased autoimmunity seen in CVID patients with mutations in the BAFF receptor transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) (70–72). Further studies are needed to refine our understanding of the complex relationship between BAFF and autoimmunity in CVID.

Other biomarkers of immune dysregulation have been linked with autoimmunity, as well as other non-infectious complications, in CVID. Importantly, an elevated T_{H1} signature has been found in the peripheral blood of these patients (73). This signature includes the prototypical T_{H1} cytokine interferon-γ (IFN-γ) and its downstream effects. This heightened T_{H1} signature in the blood is consistent with the heighted T_{H1} cellular response previously mentioned to be increased in CVID patients with autoimmune complications and elevated $CD21^{low}$ B cells. Elevated IFN-γ producing innate lymphoid cells have also been found to distinguish CVID patients with non-infectious complications (74). Thus, systemic immune dysregulation favoring T_{H1} cytokine production appears to be an important feature of CVID patients with non-infectious complications, yet the pathological basis of this skewed cytokine response remains unclear. Likewise, the therapeutic benefit of trying to neutralize this heightened T_{H1} response remains inadequately explored (**Figure 1**).

GENETICS OF CVID AUTOIMMUNITY

As CVID is a phenotypically heterogeneous disease, the expansive genetic landscape of these patients is perhaps unsurprising. While many CVID patients may have polygenic disease, recent advances in next generation sequencing (NGS) techniques have increased the discovery of monogenic forms of CVID to 15–30% of cases (75, 76) from 2–10% in 2016 (77). The majority of identified monogenic mutations encode proteins present in immune cells, which may reflect the nature of this immune disorder or a bias of the genomic analysis that is still nascent in its sophistication (77, 78). Out of the 12 monogenic mutations listed on the Online Mendelian Inheritance in Man (OMIM) database (79) in association with CVID, we will focus on those associated with autoimmunity. It is worth noting that while these mutations are associated with a clinical presentation that fits the diagnosis of CVID, many instances may not meet the full diagnostic criteria, in particular the extent of hypogammaglobulinemia typically needed. As these mutations were described in patients with CVID or CVID-like disorders and are likely to be encountered in a clinical evaluation of such patients, we include them in our discussion of CVID-associated

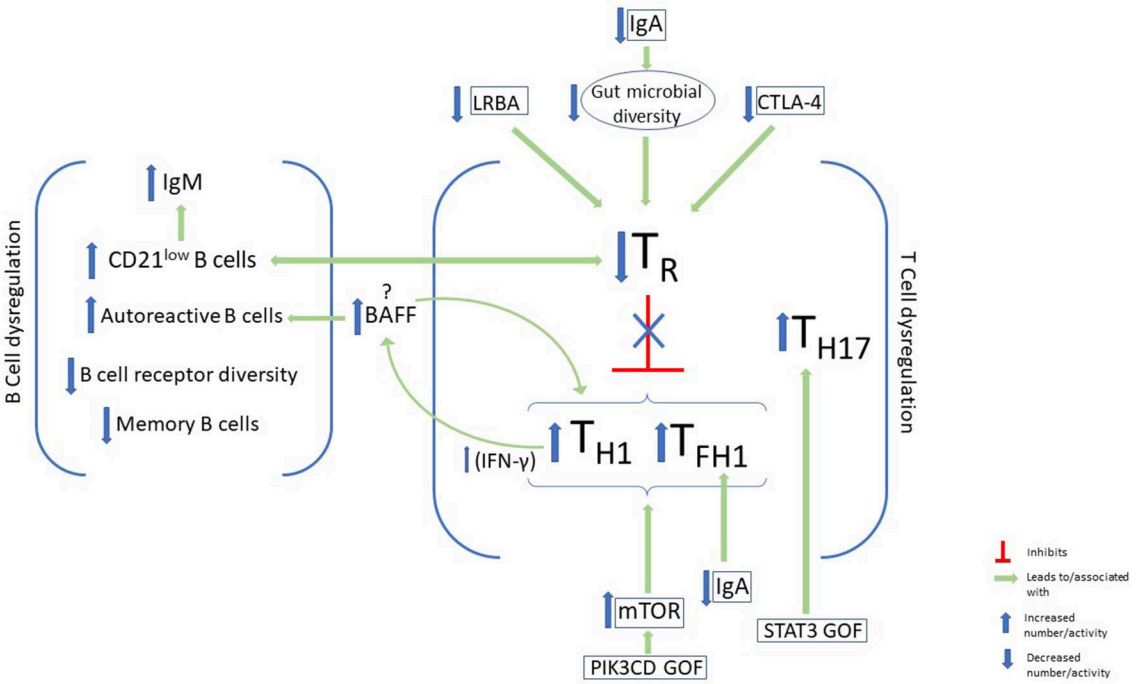

FIGURE 1 | B-cell and T-cell dysregulation and immunophenotypic characteristics of autoimmunity in CVID. Some features of B-cell dysregulation in CVID patients with autoimmunity include elevated $CD21^{low}$ B cells and IgM and increased autoreactive B cells in the periphery. On the other hand, memory B-cells, and B-cell receptor diversity are decreased. Disturbance of T-cell homeostasis, with a decrease in T regulatory cells (T_R) and a skew toward type 1 immunity, has been associated with autoimmunity in CVID. Factors that have been identified in CVID patients which lead to downregulation of T_R cells include LRBA and CTLA-4 deficiency, as well as IgA deficiency. Upregulation of mTOR activity in GOF PIK3CD may also promote this immune dysregulation, while STAT3 GOF leads to expansion of T_{H17}. BAFF augments T_{H1} cytokine production via its effects on the BAFF receptor on T cells, and promotes the survival of autoreactive antibodies via its receptor on B cells.

autoimmunity even though there is an emerging trend to categorize them separately.

Defects in the TACI, a BAFF and APRIL (a proliferation inducing ligand) receptor encoded by the *TNFRSF13B* gene, is one of the first mutations to be linked to CVID (80). It is also among the most common genetic variants found, detected in up to 10% of CVID patients who can be either heterozygous or homozygous for the mutation (81). Heterozygous TACI mutations may be more appropriately defined as a risk factor for CVID, as some are not adequately rare to be considered monogenic etiologies and are frequently found in unaffected individuals (81). Notably, CVID patients heterozygous for the *TNFRSF13B* variant have a higher risk of developing autoantibody-mediated autoimmunity than those with homozygous mutations (82). It has been hypothesized that this difference may be due to the level of dysfunction in the TACI receptor: by regulating the function of several other receptors, TACI may be involved in central B cell tolerance and that reduced function results in loss of tolerance and resultant autoimmunity. By contrast, in homozygous individuals, the complete loss of TACI function results in the inability to maintain continuous autoantibody production that would otherwise result in autoimmunity (82).

LRBA (lipopolysaccharide-responsive beige-like anchor) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) deficiencies are two closely related protein deficiencies that were detected in patients with CVID and autoimmunity (83). While mutations in *LRBA* and *CTLA4* have phenotypic variance thought to be due to incomplete penetrance and epigenetic changes, a common finding in these patients is hypogammaglobulinemia and early onset severe autoimmunity (77). CTLA-4 is an inhibitory T cell receptor that negatively regulates immunity by inhibiting excessive T cell activation and maintaining immune tolerance via its effect on T_R cells (83). LRBA, on the other hand, is thought to play a role in CTLA-4 cell surface expression, hence the phenotypic similarities in the two deficiencies (84). Deficiencies in both these proteins thus cause excessive T cell activation and breakdown of immune tolerance, resulting in autoimmunity. They are both examples of how T cell-intrinsic genetic defects can lead to hypogammaglobulinemia, further highlighting how T cell dysfunction is key to the pathogenesis of at least some cases of CVID.

Gain-of-function (GOF) mutations in *STAT3* have been identified in CVID as well as those with less profound antibody defects (75, 78). Patients with STAT3 GOF mutations also present with early-onset and quite severe manifestations of autoimmune disease (85, 86). One mechanism through which STAT3 is thought to lead to autoimmunity is by promoting the activation and expansion of autoimmunity-associated T_{H17} cells (47, 48). While a heightened T_{H1} response has been linked to CVID complications, features of these STAT3 GOF patients indicate that other forms of hyperactivated T cell responses, namely

TABLE 1 | Monogenic defects associated with autoimmunity and CVID.

Genetic defect	Protein	Immune dysregulation	Targeted treatment	Non-targeted treatment
TNFRSF13B mutation	TACI defect	Variable phenotype. Single mutation considered risk factor for CVID; also found in asymptomatic individuals		Steroids, high dose immunoglobulin, rituximab, thrombopoeitin receptor agonist
TNFRSF13C mutation	BAFF-R defect			
ICOS LOF	ICOS deficiency	Autoimmune enteropathy, cytopenias, rheumatic disease		
NFKB1 LOF	NF-κB1 deficiency	Autoimmune cytopenias and enteropathy, lymphoproliferation, lymphoma		
NFKB2 LOF	NF-κB2 deficiency	Pituitary hormone deficiencies, autoimmune disease affecting skin, hair and nails		
LRBA LOF	LRBA deficiency	Severe early-onset autoimmune disease (including autoimmune cytopenias, IBD, type 1 diabetes), lymphoproliferation, atopy (food allergy, dermatitis, urticaria)	Abatacept	
CTLA4 LOF	CTLA-4 deficiency			
PIK3CD GOF	PI3Kδ hyperactivity	Autoimmune cytopenias, primary sclerosing cholangitis, IBD, lymphoproliferation, lymphoma	Rapamycin; Leniolisib*	
STAT3 GOF	STAT3 hyperactivity	Early onset endocrine autoimmunity (type 1 diabetes, hypothyroidism), autoimmune cytopenias, lymphoproliferation, interstitial lung disease	Tocilizumab; Jakinibs	

**Currently in phase 3 clinical trial.*

T_{H17}, may also promote an autoimmune CVID phenotype. Additionally, increased STAT3 activation may impair B cell differentiation (87) leading to hypogammaglobulinemia and heightened autoreactivity found in association with CVID or more mild forms of hypogammaglobulinemia. Thus, STAT3 GOF may have both B cell-extrinsic and -intrinsic effects contributing to the immunological phenotype of affected patients.

Class IA phosphoinositide 3-kinases (PI3Ks) are heterodimeric lipid kinases that are involved in regulating cell growth, survival, and activity. Recently, a GOF mutation in the gene *PIK3CD* encoding PI3Kδ has been found in patients with CVID-like disease and autoimmunity. PI3Kδ is a PI3K subunit exclusively expressed in leukocytes. Patients heterozygous for this mutation are now said to have "activated PI3Kδ syndrome," or APDS, of which ∼200 patients have been described to date (88). Activated PI3Kδ syndrome is characterized by impaired T- and B-cell development and function, autoimmunity, and lymphoproliferation. One of the major downstream effectors of PI3K is mTOR, which regulates cell growth and survival and is critical for T_{H1} and T_{FH} cell differentiation (89, 90). While effector cells proliferate, naïve, and central memory T-cell subsets remain metabolically quiescent, likely contributing to autoimmunity, lymphoproliferation, and immunodeficiency seen in this syndrome (91). As is the case with STAT3 GOF mutations, both B cell-intrinsic and -extrinsic effects have been described as PI3Kδ is expressed in B cells and other leukocytes alike.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a family of transcription factors that are crucial for B-cell maturation, survival, differentiation, class switching, as well as self-tolerance (92). It is also a fundamental transcription factor for cytokine production by innate immune cells as well as other vital cell signaling pathways that expand beyond the immune system (93). NF-κB1 and NF-κB2 deficiencies were first described in patients of CVID affected families who were found to carry autosomal dominant mutations in *NFKB1* and *NFKB2* genes, respectively (94, 95). Mutations affecting the inducible T-cell co-stimulator (ICOS) are closely related to NF-κB deficiencies since NF-κB are activated by ICOS receptors. Because ICOS activation is essential for terminal B cell differentiation and immune tolerance (96) both ICOS and NF-κB deficiencies result in CVID-like immunodeficiency syndromes and autoimmunity (77). *NFKB1* mutation has also been linked with cytokine dysregulation, namely the elevation of type 1 cytokines that mirrors the immune profile characterizing the broader population of genetically-undefined CVID with non-infectious

complications (97). Some with *NFKB1* mutations have antibody deficiency without associated non-infectious complications (98). *NFKB2* mutations lead to autoimmunity affecting the skin, hair and nails, such as alopecia and trachyonychia, and less frequently autoimmune cytopenias, and are characterized by pituitary hormone deficiencies (99) (**Table 1**).

MICROBIOME IN CVID AND AUTOIMMUNITY

As noted above, monogenic mutations represent a minority of CVID patients with CVID. The majority of cases may be polygenic, or perhaps better defined as simply multifactorial, with environmental, epigenetic, or other factors contributing. Over the past decade, the microbiome has been implicated in the manifestation of immune dysregulation (100), and has been explored in the pathogenesis of CVID complications (46).

It has been hypothesized that impaired immunity results in increased microbial translocation across the gut barrier. This in turn drives persistent systemic immune activation leading to the disruption of the immune homeostasis (46, 101, 102). While regular immune sampling and microbial translocation occur in healthy individuals (103), the increased frequency occurring in CVID patients with diminished barrier function may lead to both local and systemic inflammation and immune dysregulation. Lipopolysaccharides (LPS) and soluble CD14 and IL-2 are used as markers for endotoxemia since their presence in systemic circulation is indicative of increased gut microbial translocation (46). CVID patients have higher levels of LPS and soluble CD14 and IL-2 compared to healthy controls, and CVID patients with autoimmune complications have higher levels compared to CVID without complications (102).

Low IgA levels are thought to play an important role in microbial dysbiosis seen in CVID since IgA is directly associated with the gastrointestinal tract immunity. Patients with isolated IgA deficiency have been found to have lower frequencies of T_R cells, especially in those patients with IgA deficiency and autoimmune disease (104). They have also been found to have reduced gut microbial diversity (105). Separately, a feedback loop has been described whereby healthy microbiota stimulate T_R cells and leads to the formation of germinal centers and IgA, which in turn maintains a healthy microbiome (106). The disruption of this feedback loop by low IgA levels and an unhealthy microbiome leads to reduction in T_R cells and subsequent immune dysregulation. Importantly, systemic IgG responses may be significant in preventing inflammation in those with IgA deficiency as a consequence of microbial dysbiosis (107). Such protective IgG responses may be significantly impaired in some CVID patients, thus predisposing to inflammation and, potentially, autoimmune disease.

Similarly to IgA deficient patients, CVID patients' gut microbial diversity was found to be reduced compared to healthy controls, with again, higher levels of soluble IL-2 and LPS detected in CVID patients with inflammatory and autoimmune complications (102). They also found an inverse correlation between T cell activation and gut microbial diversity, again suggesting that those with reduced microbial diversity have higher levels of T cell activation, and thus autoimmunity. While these findings offer insight into some drivers of immune dysregulation, further work is needed to unravel the specific mechanisms by which the microbiome affects the immune system, and how it is altered in patients with CVID, to best understand how it should be modulated or otherwise harnessed to treat complications of this immune deficiency.

TREATMENT OF AUTOIMMUNITY IN CVID

Classically, autoimmunity in CVID has been treated with broad immunosuppressive agents, including corticosteroids, methotrexate, and azathioprine among others, which place already immunodeficient patients at an even higher risk of infections. For genetically undefined CVID, the use of rituximab for autoimmune cytopenias in CVID is one of the most efficacious and safe treatments. Its use was first documented in 2004 (108), and its efficacy and safety have been well-established, especially for ITP (109). While ritixumab's efficacy with autoimmune cytopenias may be in part due to its B-cell depleting properties resulting in depletion of autoantibodies, it is thought that its success in CVID patients is also partially due to its effect on T cells (110), again highlighting the importance of T cell abnormalities in CVID. There is the documented potential risk of persistent B-cell lymphopenia after treatment with rituximab (111), but this risk is offset by the ongoing use of immunoglobulin replacement therapy. Other therapies include thrombopoietin-receptor agonists, such as romiplostim and eltrombopag which were approved by the FDA in 2008 for the treatment of cirrhosis-associated thrombocytopenia, have shown success in the treatment of thrombocytopenia in CVID and other immunodeficiencies (112, 113).

In recent years, thanks to the recent molecular and genetic findings in CVID, more targeted approaches have led to improved results through precision medicine therapy. In CTLA-4 deficiency, corticosteroids have been the most consistently used immunosuppressive agents for autoimmunity, while other steroid-sparing agents (such as mycophenolate mofetil, cyclosporine, rituximab, anti-TNF drugs) have had mixed results (114). Abatacept, a CTLA-4 immunoglobulin fusion protein, considered as CTLA-4 replacement precision therapy for these patients, has been used to treat autoimmune manifestations and shown promising results. Ten patients treated with abatacept, showed either complete resolution or partial response with regards to stabilization of their cytopenia and improvement in gastrointestinal symptoms, with no reports of adverse outcomes (114, 115). Since LRBA deficiency is related to CTLA-4 deficiency as described above, it is no surprise that abatacept has also shown similar results in LRBA-deficient patients (84). These precision therapy approaches exemplify the potential of harnessing genomics and fundamental biology to improve care of patients with PID.

STAT3 activation occurs downstream of IL-6 signaling, a cytokine implicated in autoimmune disease, such as rheumatoid arthritis (86). Thus, upon discovery of STAT3 GOF mutations, IL-6 emerged as a potential target for treatment. Tocilizumab, an IL-6 receptor antagonist, was trialed successfully in 2015 in a patient with STAT3 GOF mutation who had failed other

treatments; their T_{H17} cells which were elevated pre-treatment, similarly to what has been observed in other patients, normalized after treatment with tocilizumab (85). More recently, jakinibs, inhibitors of Janus kinases (JAKs) which are also involved in the activation cascade of STAT3, have been used adjunctly with tocilizumab in six patients and have yielded more sustained results (116). The authors of this latest study suggest that the combination of IL-6 blockade and a jakinib may be the most effective treatment strategy for patients with STAT3 GOF mutations (116).

Targeted therapy in PI3Kδ mutations have focused on the mTOR pathway and its inhibitor, rapamycin. A cohort of 26 APDS patients from the ESID registry were treated with rapamycin which showed excellent effects on the lymphoproliferative aspect of the disease, but less promising results on autoimmune cytopenias and enteropathy (117). Directed targeted inhibition of PI3Kδ is being explored with the use of leniolisib: early results of the clinical trial published in 2017 showed improvement in lymphoproliferation as well as cytopenias (118). Nemiralisib, an inhaled PI3Kδ inhibitor, has thus far shown safety and tolerability in healthy, asthmatic, and COPD patients (91) but no data has been published on APDS patients as of the publication of this review. The field of CVID has grown by leaps and bounds in recent years, coupling genomic studies with precision therapeutic approaches that have significantly improved both the efficacy and tolerability of immunosuppressive treatment for non-infectious complications.

CONCLUSION

Autoimmunity in CVID is profoundly shaped by the nature of immune dysregulation that accompanies the immune deficiency in these patients. For many years CVID patients with autoimmunity have been set apart from other CVID patients on the basis of immunophenotypic characteristics, but recent advances in our understanding of genetic defects associated with CVID have shed light on the underlying pathophysiology of autoimmunity. This improved understanding has inspired treatment of autoimmunity with targeted therapies in patients who would otherwise be subjected to broad immunosuppression. Some monogenic defects have now been listed as separate immunodeficiency syndromes, such as APDS. Importantly, many of these monogenic defects have variable clinical presentation, attributed to incomplete penetrance or variable expressivity. Additional factors, such as microbial dysbiosis, may also contribute to the pathophysiology of the disease, leading to greater heterogeneity. Future research that focuses on the immune dysregulation caused by the alteration of the gut microbiota may lead to a completely new line of therapies for these patients, such as probiotics, fecal transplantation, or even dietary recommendations. Examination of large CVID cohorts in non-Western countries may shed further light on these alternative mechanisms that may shape disease manifestations, especially given profound dietary differences and possible changes in gut microbiota between globally diverse populations. Ethnic, racial, gender, and socioeconomic factors are likely to be important to explore within Western countries. As we continue to understand the mechanisms underlying the multifactorial physiology that underlies CVID disorders, we will move closer to elucidating the fundamental immune changes that can be targeted with precision therapies to optimize disease management.

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Lack of Gut Secretory Immunoglobulin A in Memory B-Cell Dysfunction-Associated Disorders: A Possible Gut-Spleen Axis

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Background: B-1a B cells and gut secretory IgA (SIgA) are absent in asplenic mice. Human immunoglobulin M (IgM) memory B cells, which are functionally equivalent to mouse B-1a B cells, are reduced after splenectomy.

Objective: To demonstrate whether IgM memory B cells are necessary for generating IgA-secreting plasma cells in the human gut.

Methods: We studied intestinal SIgA in two disorders sharing the IgM memory B cell defect, namely asplenia, and common variable immune deficiency (CVID).

Results: Splenectomy was associated with reduced circulating IgM memory B cells and disappearance of intestinal IgA-secreting plasma cells. CVID patients with reduced circulating IgM memory B cells had a reduced frequency of gut IgA^+ plasma cells and a disrupted film of SIgA on epithelial cells. Toll-like receptor 9 (TLR9) and transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) induced IgM memory B cell differentiation into IgA^+ plasma cells *in vitro*. In the human gut, TACI-expressing IgM memory B cells were localized under the epithelial cell layer where the TACI ligand a proliferation inducing ligand (APRIL) was extremely abundant.

Conclusions: Circulating IgM memory B cell depletion was associated with a defect of intestinal IgA-secreting plasma cells in asplenia and CVID. The observation that IgM memory B cells have a distinctive role in mucosal protection suggests the existence of a functional gut-spleen axis.

Keywords: common variable immune deficiency, gut mucosal immunology, plasma cell, splenectomy, transmembrane activator and calcium-modulator and cyclophilin ligand interactor

INTRODUCTION

B cells produce antibodies of different isotypes with defined effector functions. Secretory immunoglobulin A (SIgA) is used by B cells to protect mucosal sites (1). Whereas, serum antibodies act after pathogen invasion, SIgA is localized on the external surface of epithelial cells in direct contact with commensal and pathogenic microorganisms. In the gut, SIgA regulates the diversity of the microbiota (2, 3) and modulates immune activation by enteric commensals and food antigens (4). Moreover, SIgA, by controlling intestinal homeostasis, plays a role in the prevention of bacteria-driven inflammatory, autoimmune, and neoplastic B cell pathology (5). SIgA has a similar distribution and function in the respiratory tract (6) where it prevents bacterial colonization and carriage (7). IgA exists as a monomer in the serum and as a dimer in secretions. The Joining (J) chain covalently linking two monomers forms dimeric IgA. The polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of mucosal epithelial cells binds to the J chain and transports dimeric IgA to the apical membrane. Here the external domain of the pIgR is cleaved by proteolysis thus releasing dimeric IgA bound to the fragment of pIgR called secretory component (SC) (8). In the mouse, the majority of SIgA lining the intestinal epithelium corresponds to natural antibodies, produced without intentional immunization in a thymus-independent manner by B-1a B cells. We have previously demonstrated that both B-1a B cells and SIgA are absent in the gut of asplenic mice (9). In humans, the population of B cells, known as innate IgM memory B cells, natural memory, natural effector, marginal zone B cells, is functionally similar to mouse B-1a B cells. Innate IgM memory B cells are generated from transitional B cells through Toll-like receptor (TLR) 9 stimulation *in vitro* (10–13) and can be found in patients with hyper IgM type 1 syndrome and in those with severe combined immune deficiency (14–16). While switched memory B cells are generated by previous immune responses in the germinal centers (GCs) independently from the presence of the spleen, IgM memory B cells may belong to a separate lineage (16, 17). They are found in the spleen (18) and in the peripheral blood, are generated through a T cell- and GC-independent mechanism (19), and respond to polysaccharides of encapsulated bacteria. IgM memory B cells are reduced after splenectomy (20). It has been shown that gut IgM^+ and IgA^+ plasma cells are clonally related to a large repertoire of IgM memory B cells disseminated throughout the intestine (21). In the intestine, IgA class switching is mediated by two different mechanisms, one dependent and one independent on T cells. T-cell dependent SIgA is generated by the adaptive immune response in the GCs of mesenteric lymph nodes and Peyer patches (22). IgA class switch can occur in a T cell-independent manner in the lamina propria (23, 24) and in the gut-associated lymphoid tissue (25, 26), as demonstrated in patients with CD40 ligand deficiency (23). In T cell-independent IgA class switch (27, 28), an important role is played by the interaction between the transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and its ligand a proliferation inducing ligand (APRIL) (29). This phenomenon occurs in a MyD88/IRAK4-dependent manner (30). Here, we investigate the gut mucosa of two distinct clinical conditions only sharing the reduction of circulating IgM memory B cells, i.e., splenectomized patients and patients affected by CVID (31). We show that patients with low numbers of circulating IgM memory B cells have a reduced frequency of IgA^+ plasma cells in the gut and a disrupted film of SIgA on epithelial cells. We also show that *in vitro* IgM memory B cells are the only B cell type able to respond to TLR9 and TACI cross-linking by differentiating into IgA^+ plasma cells.

Abbreviations: AID, activation-induced cytidine deaminase; APRIL, a proliferation inducing ligand; CVID, common variable immune deficiency; HD, healthy donors; IL, interleukin; J, Joining; pIgR, polymeric immunoglobulin receptor; SIgA, secretory immunoglobulin A; SC, secretory component; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; TLR, Toll-like receptor.

RESULTS

Intestinal Secretory Immunoglobulin A Is Reduced After Splenectomy

We and others have previously shown that removal of the spleen causes the reduction of IgM memory B cells in the peripheral blood (12, 20, 32). In order to verify whether IgM memory B cells might have a role in the mucosal protection, we analyzed duodenal biopsies of seven patients who had been splenectomized because of traumatic rupture of the spleen and did not show any pre-existing immune, hematologic, or neoplastic comorbidities. They underwent upper endoscopy to investigate dyspepsia. All of them had serum Ig levels within the normal range (**Supplementary Table 1**). The number of $CD27^+$ IgM and switched memory B cells was reduced in comparison to healthy donors (HD, $n = 51$). Absolute counts for $CD27^+$ IgM^+ B cells were 17 ± 11 cells/mm^3 (normal value 55 ± 35 cells/mm^3, $p = 0.003$), while absolute counts for $CD27^+$ switched memory B cells were 29 ± 17 cells/mm^3 (normal value 58 ± 37 cells/mm^3, $p = 0.6$) (**Supplementary Table 1**). Cryostat sections stained with phalloidin, in order to visualize the tissue architecture, and with antibodies against IgA, were analyzed by confocal microscopy. In the HD cohort, IgA^+ plasma cells appeared as bright and large green cells in the axis of the villi and beneath the epithelial cell layer in the crypts (**Figure 1A**, IgA panel). SIgA was transported through the epithelial cells to the luminal surface where it remained in the mucus. IgA transport can be tracked by staining the SC with a specific antibody. The pIgR fragment became visible toward the luminal side of the epithelial cells after the enzymatic cleavage that released the SC bound to IgA into the lumen while directing the rest of the pIgR to the recycling pathway (**Figure 1A**, SC panel). The J chain was only detected in the mucus because the epitope identified by the antibodies we used was not accessible either in plasma cell cytoplasm or when the J chain was bound to the intact pIgR (**Figure 1A**, J chain panel). Furthermore, HD IgM^+ plasma cells were visualized as bright and large blue cells in the axis of the villi and beneath the epithelial layer in the crypts, while secretory IgM (SIgM) was

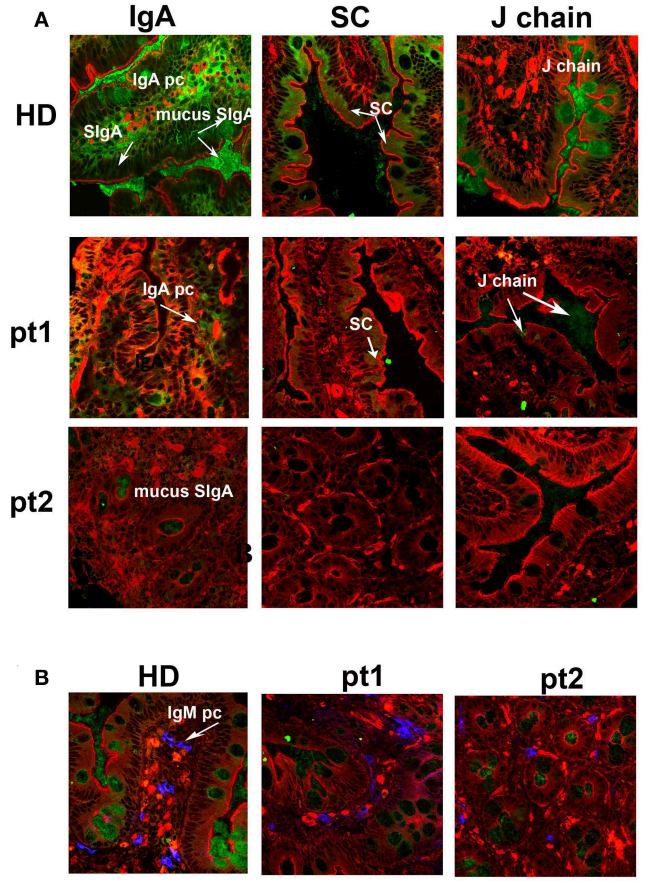

FIGURE 1 | Secretory immunoglobulin A (SIgA) is reduced in splenectomized patients. **(A)** Cryostat sections of the duodenum were stained in red with phalloidin (a toxin that binds and stains filamentous actin) and in green with antibodies against either IgA (left panels), secretory component (SC, middle panels), or J-chain (right panels). Biopsies of one healthy donor (HD) and two representative splenectomized patients (pt.1 and pt.2) are shown. A reduction of IgA^+ plasma cells, SIgA, SC, and J chain is observed after removal of the spleen. **(B)** IgM^+ plasma cells are shown in blue along the axis of the villi in HD and asplenic patients. IgM is not significantly secreted.

not evident at the luminal side of the epithelial cells (**Figure 1B**, IgM panel). We counted plasma cells in the villous axis in well-oriented duodenal biopsies by considering seven villi per patient in three different slides. In splenectomized subjects, intestinal IgA^+ plasma cells were reduced to half of normal (6.8 ± 4.0 vs. 15.0 ± 0.7 plasma cells/villus, $p < 0.0001$), and SIgA was not significantly transported through the epithelial cells. In some cases, patches of SIgA scattered in the lamina propria were observed (**Figure 1A**, IgA panel). Both SC and J chain were hardly visible (**Figure 1A**, SC and J chain panels). IgM was not significantly transported to the luminal side of the epithelial cells, and IgM^+ plasma cells were slightly reduced in splenectomized patients (**Figure 1B**, IgM panel). Thus, removal of the spleen was associated not only with a reduction of circulating IgM memory B cells, but also with the disappearance of the SIgA film on epithelial cells.

Intestinal Secretory Immunoglobulin A Are Reduced in Common Variable Immune Deficiency Patients With Circulating Immunoglobulin M Memory B Cell Depletion

We have previously shown that in CVID patients the frequency and numbers of circulating switched memory B cells are always reduced, but IgM memory B cells may be preserved (31). Patients with a severe defect of IgM memory B cells suffer from recurrent respiratory infections mostly caused by encapsulated bacteria, with a possible evolution in chronic lung diseases (33). In contrast, CVID patients with normal frequencies of IgM memory B cells have a lower incidence of infections. Moreover, serum IgA has a protective role against respiratory infections as demonstrated by the increased risk of pneumonia in CVID patients with IgA serum levels lower than 7 mg/dl (33). Here, we analyzed a cohort of 33 CVID patients and 51 HD. We stratified CVID patients according to the number of peripheral IgM memory B cells. Group 1 (22/33) had an absolute number of IgM memory B cells <20 cells/mm^3 (6 ± 6 cells/mm^3), whereas Group 2 (11/33) had a number of IgM memory B cells >20 cells/mm^3 (105 ± 110 cells/mm^3).

Switched memory B cells were reduced in both groups, with Group 1 showing significantly lower levels than Group 2 (2 ± 3 cells/mm^3 vs. 17 ± 24 cells/mm^3, $p = 0.006$). Serum IgM concentration was significantly lower in Group 1 (25.5 ± 68.3 mg/dl) as compared to Group 2 (30.8 ± 19.6 mg/dl, $p = 0.009$). Serum IgA levels were also reduced in Group 1 in comparison to Group 2 (5.4 ± 11.8 mg/dl vs. 23.1 ± 60.7 mg/dl), but the difference did not reach significance ($p = 0.06$). Serum IgG levels at diagnosis (208.4 ± 130.4 vs. 267.7 ± 118.4 mg/dl) were comparable (**Supplementary Table 2**).

The analysis of levels of specific anti-pneumococcus capsular polysaccharide (PCP) IgM and IgA before and after pneumococcal vaccination revealed no difference before immunization between Groups 1 and 2 for both specific anti-PCP IgM (3.6 ± 8.8 vs. 8.9 ± 9.7 UI/ml, $p = 0.2$) and IgA (2.1 ± 3.1 vs. 4.2 ± 4.3 UI/ml, $p = 0.2$). Post immunization anti-PCP IgM and IgA were reduced in both groups in comparison to HD ($p < 0.0001$). Group 1 showed significantly lower anti-PCP IgM (2.3 ± 3.7 UI/ml) than Group 2 (12.2 ± 12.6 UI/ml, $p = 0.02$). Group 1 post anti-PCP IgA was lower (1.4 ± 1.7 UI/ml) than Group 2 post anti-PCP IgA (92.2 ± 172.8 UI/ml), even if this difference was not statistically significant due to the high SD (**Supplementary Table 2**).

The clinical picture of the two groups was different, with Group 1 displaying a more severe phenotype. Recurrent pneumonia episodes and bronchiectasis were reported more frequently in Group 1 than in Group 2 (59 vs. 9%, $p = 0.009$ and 73 vs. 27%, $p = 0.02$, **Supplementary Table 3**). Gastrointestinal symptoms were also described more commonly in Group 1 than in Group 2 (63 vs. 18%, $p = 0.02$). In particular, recurrent episodes of diarrhea were reported in 59% of patients from Group 1 and in 18% from those in Group 2 ($p = 0.03$). Detailed gastrointestinal diagnosis and gut alteration have been summarized in **Supplementary Table 3**.

Patients from Group 1 had frequently more splenic alteration in comparison to those in Group 2 (77 vs. 36%, $p = 0.05$). Interestingly, subjects who had severe splenomegaly (six patients) or who had been splenectomized (four patients) were all classified as Group 1 (**Supplementary Table 3**). Patients with splenomegaly showed lower number of IgM memory B cells and switched memory B cells in comparison to those without spleen alteration as summarized in **Supplementary Figure 1**.

It has been widely recognized that gastrointestinal infections and chronic intestinal inflammation are common in CVID (34–36) and are associated with high morbidity (37). Moreover, CVID patients have an increased risk of gastric cancer (38), and therefore surveillance gastroscopy with collection of biopsies is regularly performed in our center according to the Italian guidelines (www.ipinet.org). We excluded from the study CVID patients with a histological report of an abnormal tissue architecture (i.e., villous atrophy). We analyzed the presence of IgA^+ plasma cells, IgM^+ plasma cells, and the distribution of SIgA and sIgM in the duodenal samples of the two CVID groups (**Supplementary Table 2**). We counted plasma cells in the villous axis in well-oriented duodenal biopsies by considering seven villi per patient in three different slides. Only one patient of Group 1 had IgA^+ plasma cells in the gut, whereas IgA^+ plasma cells were visible in eight patients (73%) of Group 2 (**Figure 2**). Only one patient of Group 1 (1/22, 4.5%) had IgM^+ plasma cells in the intestine, whereas IgM^+ plasma cells were visible in six patients (6/11, 54.5%) of Group 2 (**Figure 3**, IgM, middle panel). No patient of Group 1 had SIgA in the intestine, whereas SIgA were found in the same patients of Group 2 who had IgA^+ plasma cells (**Figure 2**). In only two patients of Group 2, sIgM were detectable in the microfilm of mucus on epithelial cells (**Figure 3**, IgM, lower panel). In summary, we found that a low number of circulating IgM memory B cells were associated with a severe defect of mucosal SIgA.

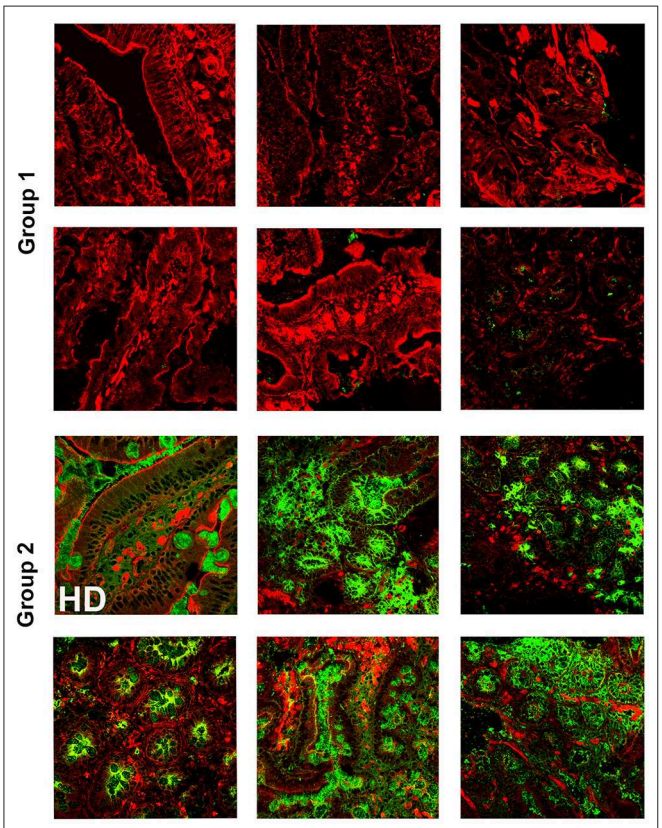

FIGURE 2 | Lamina propria expression of secretory immunoglobulin A (SIgA) in the gut of CVID patients who have reduced (Group 1) or normal (Group 2) number of circulating immunoglobulin M (IgM) memory B cells. Cryostat duodenal sections were stained with phalloidin (red) and immunoglobulin A (IgA) (green) in six patients of Group 1 with reduced numbers of circulating IgM memory B cells **(upper panels)**, and a control subject (HD) together with five patients belonging to Group 2 with normal numbers of circulating IgM memory B cells **(lower panels)**.

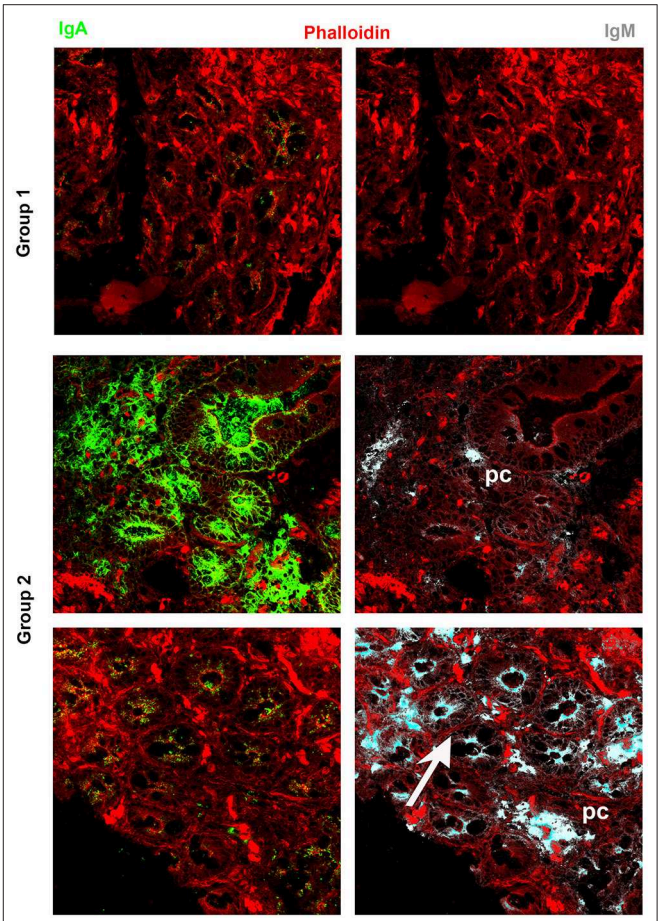

FIGURE 3 | Immunoglobulin M (IgM) can substitute immunoglobulin A (IgA) in the gut of common variable immune deficiency (CVID) patients. Cryostat sections were stained with phalloidin (red), IgA (green), and IgM (gray). IgA **(left panels)** and IgM **(right panels)** staining in the same sections are shown separately. In CVID patients belonging to Group 1, neither IgA nor IgM is expressed. In most CVID patients belonging to Group 2, IgA is the major isotype expressed and transported (pt.22, as representative example). IgM substitutes IgA in one case (pt.30, **lower panel**) and is transported through epithelial cells.

Toll-Like Receptor 9 and Transmembrane Activator and Calcium-Modulator and Cyclophilin Ligand Interactor Cooperatively Induce Class Switching of Immunoglobulin M Memory B Cells to Immunoglobulin A

Transitional, mature-naïve and memory B cells all express TACI on the cell-surface. The highest expression is observed in IgM memory B cells and the lowest in switched memory B cells from peripheral blood (**Supplementary Figure 2A**). Stimulation of TLR9 through its ligand CpG upregulates TACI expression on naïve B cells and IgM memory B cells. TACI expression remains low in switched memory B cells and undetectable on newly generated plasma cells (**Supplementary Figure 2B**). We asked whether all B cell types are able to switch to IgA upon TACI engagement and investigated the effects of co-stimulation with the TLR9 ligand CpG. The response of B cell populations to CpG had been previously investigated (10). IgM memory B cells proliferate and differentiate into plasma cells secreting high amount of IgM and barely detectable IgG, but never IgA. Mature-naïve B cells survive without differentiation. Transitional B cells proliferate and differentiate into IgM memory B cells and IgM^+ plasma cells (10, 13). In most normal adults, the population of switched memory B cells is composed of 2/3 by IgG expressing memory B cells with the remaining 1/3 expressing IgA. All switched memory B cells expand upon stimulation with CpG and differentiate into IgG^+ and IgA^+ plasma cells (39). Cooperation between TLR9 and TACI is possible because the two receptors share the same adaptor protein, MyD88 (30). Moreover, APRIL, the ligand for TACI, is expressed in the gut (22, 24), where the microbiota provides TLR ligands in abundance. We studied the effects of TACI or TACI in combination with CpG in mature-naïve ($CD19^+$ $CD24^+$ $CD27^-$) or IgM memory B cells ($CD19^+$ $CD24^+$ $CD27^+$ IgG^- IgA^-) sorted from the peripheral blood of adult donors. In all cultures, we included interleukin IL-4 and IL-21, a combination of cytokines that increases survival of mature-naïve B cells and plasma cell formation without changing the quality of the response. Plasma cells can be distinguished from memory B cells based on their higher CD27 expression and can express either IgM (IgM plasma cells) or other isotypes (switched plasma cells). After 7-day culture with anti-TACI, neither mature-naïve nor IgM memory B cells generated plasma cells (**Figure 4A**). A small fraction of mature-naïve B cells and a large one of IgM memory B cells switched to IgA. This is suggested by the loss of surface IgM (**Figure 4A**) and demonstrated by the expression of intracellular IgA in 0.5% of the mature and in 28% of the IgM memory populations (**Figure 4C**, TACI+IL-4+IL-21). When CpG was added to the cultures, IgM memory B cells differentiated into IgM^+ and IgM^- plasma cells (**Figure 4B**, TACI+CpG+IL-4+IL-21) containing high amounts of intracellular IgM or IgA. Only few IgM^+ (4%) and even less IgA^+ (0.1%) plasma cells were obtained from mature B cells (**Figure 4C**) in the same conditions. Transitional, mature-naïve and IgM memory B cells stimulated with CpG, anti-TACI and IL-4/IL-21 all produced IgM. IgA was detected only in the cultures containing IgM memory B cells (**Figure 4D**). In summary, we concluded that only IgM memory B cells differentiate into IgA^+ plasma cells in response to TACI and CpG.

Expression of a Proliferation Inducing Ligand, CD27, and Immunoglobulin M in the Adult Gut

Recently, Magri et al. (21) demonstrated that IgM^+ plasma cells in the gut are clonally related to IgM memory B cells disseminated throughout the intestine. They also showed that IgM memory B cells switch to IgA in response to T-independent and dependent signals *in vitro*. We asked the question whether IgM memory B cells expressing TACI could be found in the intestine in the vicinity of the TACI-ligand APRIL. We showed that in the adult gut, APRIL can be detected in the epithelium, and it is expressed by crypt epithelial cells but is not equally expressed by each epithelial cell, probably reflecting the topographic distribution of inductive signals. We observed a gradient of APRIL expression starting at the luminal side and progressing toward the basal side of the epithelial cell (**Figure 5**). B cells were localized under the epithelial cell layer where APRIL was extremely abundant. In addition, IgM^+ B cells expressed TACI (**Figure 6**). An indication that B cells follow a migratory pathway connecting spleen and intestine has been suggested before based on the metastatic behavior of marginal zone lymphomas, as also shown in one of our CVID patients who developed a marginal zone B-cell lymphoma of the spleen, a tumor thought to originate from memory B cells expressing CD27 and IgM. We observed large numbers of $CD27^+$ IgM^+ B cells in the intestinal biopsy of this CVID patient of Group 1. Differently from the organized distribution localized around the cryptae and along the axis of the villi observed in HD, in the intestinal biopsies taken before splenectomy, clusters of $CD27^+$ IgM^+ cells were observed throughout the tissue, infiltrating and disrupting the structure (**Supplementary Figure 3**).

DISCUSSION

SIgA is a pillar of the mucosal barrier against bacterial dissemination, it prevents adhesion and penetration of antigens, and neutralizes biologically active substances and viruses forming complexes in antigen- and non-antigen specific manner by interacting with IgA Fc receptors (40, 41). After bacterial sampling by dendritic cells or invasion, pathogens transported to the local lymph nodes induce the formation of GCs. Highly specific B cells then migrate back to the submucosa into the Peyer's patches and here differentiate into IgA-producing plasma cells. In the lamina propria, B cells may also switch into IgA in a T cell-independent way (21). The T cell-independent generation of SIgA by B cells may represent a mechanism to generate IgA expressing a wide repertoire of Ig genes, useful to face the thousands of different bacterial species of the microbiota (23, 24, 42), and to control host-microbiota mutualism, reducing the risk of bacterial translocation and immune activation (43–45). IgM memory B cells have been shown to home to the gut and to locally switch to IgA (21, 46). Since IgM memory B cells are reduced in patients who had been

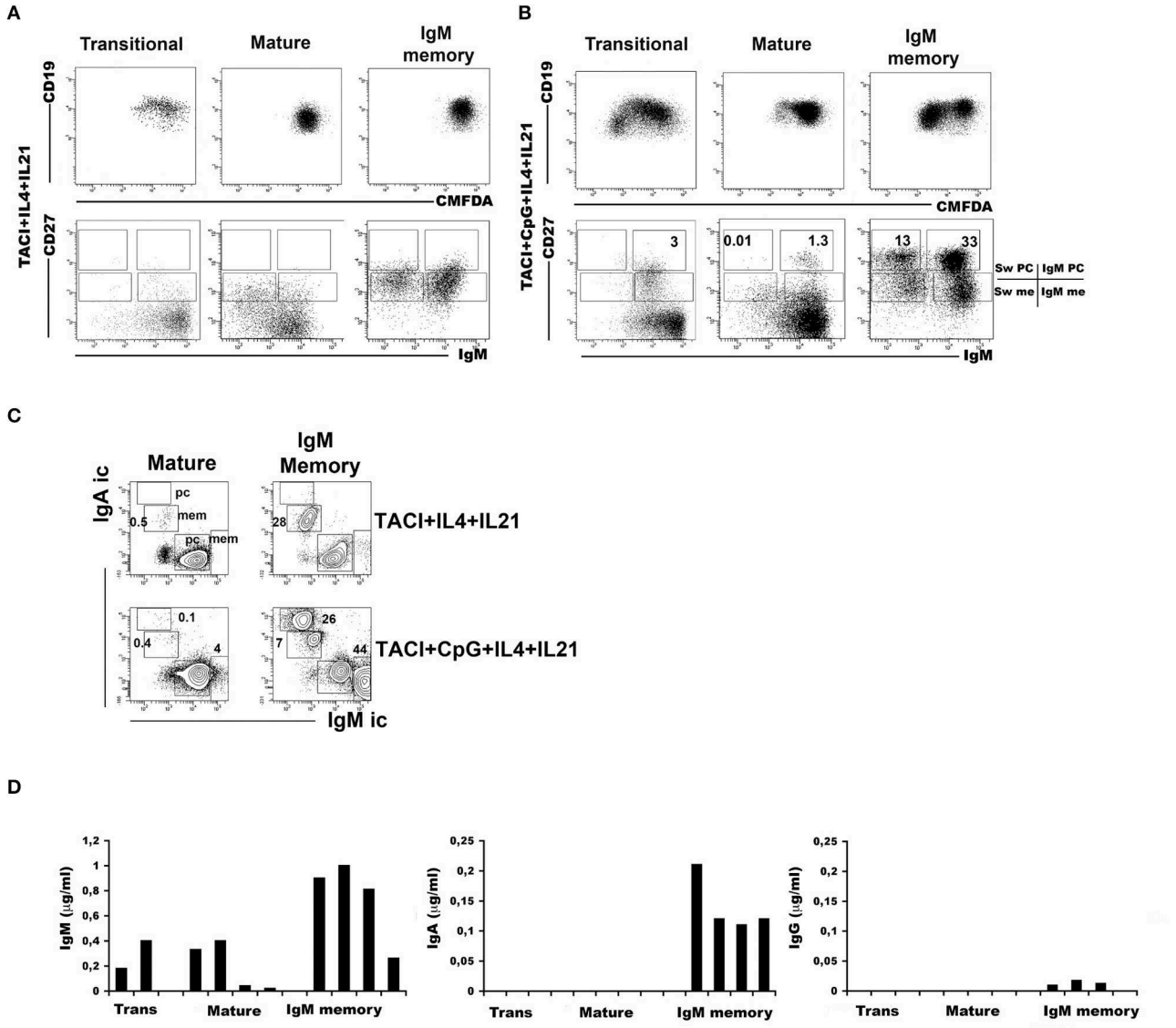

FIGURE 4 | Immunoglobulin M (IgM) memory B cells differentiate into IgA$^+$ plasma cells in response to transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and toll-like receptor 9 (TLR9) co-stimulation. Flow-cytometry analysis after 7 days in culture with anti-TACI plus IL-4 and IL-21. The loss of intracellular CMFDA tracks cell division. **(A)** No proliferation is observed in mature-naive and IgM memory B cells, whereas a modest expansion of transitional B cells is observed (upper panels). The IgM/CD27 staining (lower panels) shows the loss of surface IgM in 17.0% of mature-naïve and 43.6% of IgM memory B cells (bottom panels). **(B)** The addition of CpG induces proliferation as shown by the loss of CMFDA fluorescence, mainly in transitional and IgM memory B cells (top panels). Differentiation into IgM$^+$ CD27$^+$ plasma cells is observed in all B cell types. Switched plasma cells are only generated from IgM memory B cells. **(C)** After 7 days with anti-TACI, a small fraction of mature-naïve B cells had switched to IgA. The addition of CpG further increased survival. Upon TACI cross-linking, almost half of the IgM memory B cells switched to IgA, but when CpG was added to the culture, IgM memory B cells differentiated into IgA$^+$ plasma cells. **(D)** Graphs indicate the concentration of IgM and IgA (ng/ml) detected by ELISA in culture supernatants obtained from B cells stimulated with anti-TACI, CpG, IL-4, and IL-21. IgM can be detected in the supernatants of all B cell types, but only stimulated IgM memory B cells secreted IgA. IgG production was never detectable.

splenectomized or are affected by CVID (20, 31), we asked the question of whether the reduction of this B cell subset might influence the production of SIgA in the gut. We analyzed patients with an intact and functional immune system that had been splenectomized because of injury and patients affected by CVID, a primary antibody deficiency. In splenectomized patients, the T cell-independent responses to polysaccharide antigens were impaired (47) but the adaptive immune response in the lymph nodes remained functional and serum Ig levels were normal. We showed that splenectomy does not affect the number of peripheral naive B, but causes a reduction of IgM memory B cells, as previously shown (12, 20, 48). The reduction of IgM memory B cells in the peripheral blood was associated with the reduction of IgA$^+$ plasma cells in the gut. In particular, our patients did not

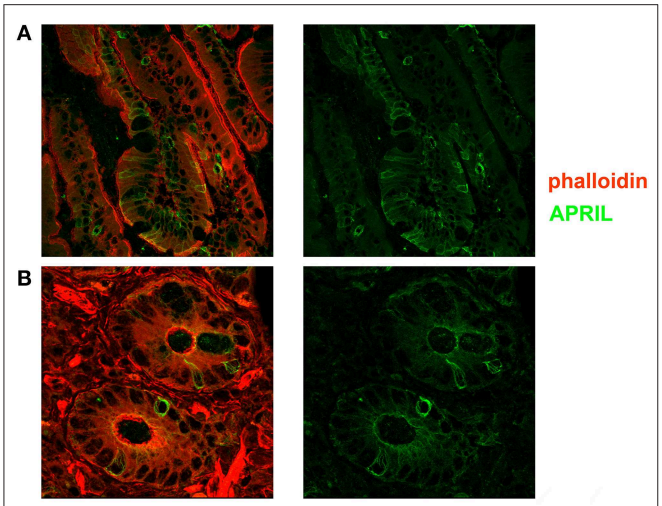

FIGURE 5 | Expression of a proliferation inducing ligand (APRIL) in the adult gut. Cryostat sections from adult intestine were stained for actin (phalloidin, red) and APRIL (green). **(A)** We show the overlay of phalloidin and APRIL staining (left) and APRIL alone (right). APRIL can be detected in the epithelium, but is not equally expressed by each epithelial cell, probably reflecting the topographic distribution of inductive signals (magnification ×60). **(B)** Details of cryptae analyzed with ×60 objective amplification are shown. We observe a gradient of APRIL expression starting at the luminal side and progressing toward the basal side of the epithelial cell.

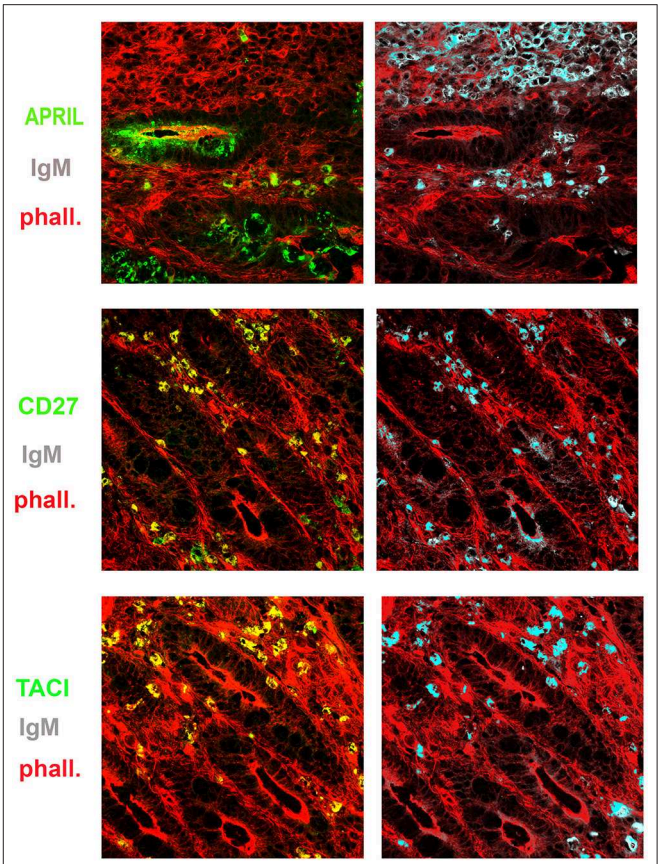

FIGURE 6 | Expression of a proliferation inducing ligand (APRIL), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), CD27, and immunoglobulin M (IgM) in the gut. Cryostat sections from the intestine were stained for actin (phalloidin, red) and APRIL (green) **(upper panel)**. APRIL is expressed by epithelial cells of the crypts. Intestinal sample is stained for CD27 (green) and IgM (gray) **(middle panel)**. From the same field (×20 magnification) we show in separate panels the overlay of phalloidin, TACI, and IgM. $CD27^+$ (left) IgM^+ (right) B cells were distributed among the crypts (middle panels). IgM^+ B cells express TACI **(lower panel)**.

have pre-existing diseases, and splenectomy was due to traumatic rupture of the spleen. If SIgA in the intestine was generated by local immune responses initiated either by transitional or naïve B cells, a reconstruction of the SIgA layer should be observed after splenectomy. This did not happen even years (few months to 15 years) after splenectomy. Thus, the responses occurring in the organized lymphoid tissue of the gut and in the lymph nodes throughout the body were not sufficient to fully re-construct the continuous film of SIgA in the gut. Of note, these findings may provide a pathophysiological explanation for the gut microbiota alterations over time after splenectomy and the higher susceptibility observed in splenectomized patients to infections sustained by enteric bacteria, including *Enterococci*, *Escherichia coli* (*E. coli*), and other enterobacteria (49, 50).

Patients with CVID had reduced serum Ig levels and were unable to mount an effective antibody response (37). Antibodies of IgG isotype are regularly administered in order to transfer the antigenic experience of donors. However, despite IgG replacement, the subset of CVID patients having reduced frequency of IgM memory B cells, reduced number of switched memory B cells and a very low IgA level had a severe infection risk (33). Moreover, CVID patients suffered from infectious and inflammatory disorders (34, 36), all conditions affecting patients' morbidity and quality of life (51). In CVID patients, the shift in the gut microbial composition and the reduced diversity of the gut microbiota promoted immune activation and inflammation, without obvious associations with antibiotic use (52). Spleen involvement was frequent in CVID. Splenomegaly had been reported in one out of four patients enrolled in the database of the European Society for Immunodeficiencies (ESID), but neither its causes nor its consequences were well-understood. Splenomegaly is associated with autoimmunity, granulomas but also with liver disease and portal hypertension (53). Here, we observed that patients with splenomegaly had reduced IgM memory and switched memory B cells, confirming data from other cohorts (54, 55).

The majority of CVID patients with a normal number of IgM memory B cells had a visible layer of SIgA, while all CVID patients with low numbers of IgM memory B cells lacked SIgA. The observation that at least one of our patients could substitute SIgA and IgA^+ plasma cells with sIgM and IgM^+ plasma cells may explain why selective IgA deficiency can remain asymptomatic. T cell-independent IgA class switch is promoted by the interaction between TACI and its ligand

APRIL through up-regulation of AID expression (27–29). We show that IgM memory B cells are able to develop into IgA^+ plasma cells *in vitro* upon stimulation with TLR9 and TACI. TACI, differently from other members of the TNF receptor family, is able to cooperate with TLR9. Protection of mucosal sites from colonizing bacteria has been a prerequisite for life throughout evolution. The innate immune system evolved first. B and T cells appeared in fish. Fish have no bone marrow, lymph nodes or GCs, but they have the spleen and in the gut they produce an antibody called IgT, dimeric as IgA and transported to the intestinal lumen by the pIgR (56, 57). Mice have SIgA generated by B-1a B cells, independently of T cells. It is attractive to hypothesize that a primitive defense system still exists in man, and IgM memory B cells belong to it. Further studies are necessary to identify the cellular and molecular mechanisms used by colonizing bacteria to trigger the development of IgM memory B cells in the spleen, their migration to the gut, and the organization of local immunity. Recently, it has been demonstrated that IgM^+ plasma cells in the gut are clonally related to IgM memory B cells disseminated throughout the intestine (21), and that IgM memory B cells switch to IgA in response to T-independent and dependent signals *in vitro*. We confirm that TACI-expressing IgM memory B cells are localized under the epithelial cell layer. Epithelial cells, in turn, express the TACI ligand APRIL.

In conclusion, our results suggest that IgM memory B cells may play a distinctive role in mucosal protection by migrating to the gut where they switch to IgA. In the absence of IgM memory B cells, naïve or transitional B cells, which are both present and functional in our group of splenectomized patients, are not able to regenerate the SIgA film and to replenish the IgA plasma cells in the gut. New tools should be developed in order to substitute the function of SIgA in asplenia and in primary antibody deficiencies, and further studies are necessary to confirm the existence of a functional gut-spleen axis.

MATERIALS AND METHODS

Patients

Seven patients, who had been splenectomized for trauma, without pre-existing comorbidities were enrolled into the study as they had undergone an upper endoscopy for investigating a dyspepsia. Patients affected by CVID ($n = 33$) were diagnosed according to the ESID/PAGID criteria (26). Patients with CVID have an increased risk of gastric cancer, and for this reason the Italian guidelines include annual endoscopy with biopsy collection. All patients were on intravenous or subcutaneous immunoglobulin substitution therapy with trough IgG serum levels above 500 mg/dl according to the national guidelines. CVID participants could also be treated with additional drugs following consolidated clinical practice and guidelines. However, no patient was receiving either steroid treatment or immunosuppressive therapy. None of the patients included in this study had TACI mutations, and all CVID patients with architectural abnormalities of the duodenal mucosa at the study time were excluded from the study. Fifty-one HD were enrolled as controls for blood values and 15 HD for intestinal biopsies. For 19 patients and 20 HD we assessed by ELISA test the levels of specific anti PCP-IgM and anti PCP-IgA before and after pneumococcal vaccination by one dose of a 23-valent polysaccharide vaccine (Pneumovax®) as described by Cavaliere et al. (58). The choice to measure IgM and IgA anti-PCP instead of IgG, was due to the necessity to overcome the impossibility of studying vaccine responses in subjects on IgG replacement therapy. Protocols were conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee.

Flow Cytometry Analysis

Peripheral blood mononuclear cells were isolated and stained as previously described (12). Briefly, mature-naïve B cells are ($CD24^+CD27^-IgM^+IgD^{bright}$). Two populations of memory B cells can be identified: IgM memory B cells ($CD24^+CD27^+IgM^+IgD^{dull}$) and switched memory B cells ($CD24^+CD27^+IgM^-IgD^-$).

Cell Sorting

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Paque™ Plus (Amersham Biosciences, Little Chalfont, UK) density-gradient centrifugation and were stained with the following antibodies: clone ML5 (anti-CD24), clone M-T271 (anti-CD27), clone G18-145 (anti-IgG), and streptavidin-APC-Cy7 were obtained from BD Biosciences (San Diego, CA, USA) and clone B35C6B4 (anti-IgA_1) and clone A964D2 (anti-IgA_2) from Southern Biotechnologies. After staining the lymphocytes were sorted as mature-naive B cells ($CD24^+CD27^-$) and IgM memory B cells ($CD24^+CD27^+IgG^-IgA^{1-2-}$) using a FACSvantage SE (Becton and Dickinson, Sunnyvale, California, USA). A negative gating strategy was used to sort IgM memory B cells in order to avoid B-cell activation through the BCR. Dead cells were excluded from analysis by side/forward scatter gating and cell purity was >98%. Cord blood mononuclear cells were stained for CD24 and CD38 and sorted for transitional B cells ($CD24^{bright}CD38^{bright}$) as described above.

Cell Culture

Transitional, mature naive, and IgM memory sorted B cells were labeled with 5-Chloromethyl fluorescein diacetate at the final concentration of 0.1 mg/ml (CellTracker CMFDA, Molecular Probes, Eugene, OR) and cultured in 96 well plates (Becton Dickinson) with RPMI 1640 (Gibco BRL), 10% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories Logan UT), 2% L-glutamine (Gibco BRL), $5 \times 10–5$ M 2-βmercaptoethanol (Sigma, St. Louis, USA) and 20 mg/ml gentamycin (Gibco BRL), supplemented with either 2.5 μg/ml CpG-ODN17 (Hycult Biotechnology, Uden, The Netherlands), 20 ng/ml IL-21 (Peprotech, UK), 20 ng/ml IL-4 (Peprotech, UK), 1 μg/ml anti-human CD267 (anti-TACI) (eBioscience, San Diego, CA, USA), and beads coated with anti-mouse IgG (Dynal Life Technologies Europe) at the proportion of one bead per 50 cells. Cell proliferation and phenotypic analysis were performed on day 7 by flow-cytometry using a FACSCalibur Flow Cytometer

(BD Biosciences). Secreted Igs were detected in the supernatants at day 7 by ELISA.

Enzyme-Linked Immunosorbent Assay

Briefly, 96-well plates were coated overnight with purified goat anti-human IgA, IgG, or IgM antibodies (Jackson Immuno Research Laboratories, Pennsylvania, USA). After washing and blocking, plates were incubated for 1 h with the supernatants of the cultured cells. After washing, plates were incubated for 1 h with peroxidase-conjugated fragment of goat antihuman IgA, IgG or IgM antibodies (Jackson Immuno Research Laboratories), and the assay was developed with o-phenylendiamine (Sigma-Aldrich). Optical density was measured on a microtiter plate reader at 450 nm and Ig concentrations were calculated by interpolation with the standard curve.

Confocal Microscopy

Intestinal tissues were collected and immediately frozen in liquid nitrogen, and kept at −80°C until the time of the study. Multiple 5 μm cryostat sections obtained from frozen samples, included in cryostat embedding medium (Bio-Optica, Milan, Italy) were fixed in cold acetone, washed with PBS (Sigma, St. Louis, MO Sigma) and incubated for 45 min with Phalloidin-TRITC (70 mM, Sigma). Reagents used in the different stainings were FITC-anti-human- IgA, FITC-anti-human secretory component (Nordimmune, Tilburg, the Netherlands), FITC-antihuman J-chain (Mc19-9) (Serotec), anti-human CD256 (anti-APRIL, clone T3-6) (BioLegend, San Diego, CA, USA), anti-human CD267 (anti-TACI, clone 11H3) (eBioscience), TRITC-goat antihuman IgM, μ chain specific (Jackson Immunoresearch), and FITC-anti-human CD27 (clone MT271) obtained from BD Biosciences. Intestine sections were analyzed in a confocal microscope (Olympus FV1000), and images were acquired at ×20 and ×60 objective amplification. Three slides per patient were analyzed at the confocal microscope by four independent experts.

Statistical Analysis

For comparison of clinical and biomarker changes between groups, the Student *T*-test and Mann-Whitney test were used for parametric or non-parametric datasets. Data were analyzed in the StatView statistical MacIntosh program (StatView Software, San Diego, CA). A level of $p < 0.05$ was considered statistically significant.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Rome, La Sapienza. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RC, IQ, AD, MR and GC designed and coordinated the study, interpreted data, and wrote the manuscript. SC, EP, CM, OG, AA, EG and PB followed-up patients over time, locally collected data, made experiments, did statistical analysis, and reviewed the paper for final approval. All authors significantly participated in the drafting of the manuscript, critical revision of the manuscript for important intellectual content, and provided approval of the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02937/full#supplementary-material

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Histocompatibility Complex Status and Mendelian Randomization Analysis in Unsolved Antibody Deficiency

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The pathogenesis in the majority of patients with common variable immunodeficiency (CVID), the most common symptomatic primary immunodeficiency, remains unknown. We aimed to compare the minor and major histocompatibility complex (MHC) markers as well as polygenic scores of common genetic variants between patients with monogenic CVID and without known genetic mutation detected. Monogenic patients were identified in a CVID cohort using whole exome sequencing. Computational full-resolution MHC typing and confirmatory PCR amplicon-based high-resolution typing were performed. Exome-wide polygenic scores were developed using significantly different variants and multi-variant Mendelian randomization (MR) analyses were used to test the causality of significant genetic variants on antibody levels and susceptibility to infectious diseases. Among 83 CVID patients (44.5% females), monogenic defects were found in 40 individuals. Evaluation of the remaining CVID patients without known genetic mutation detected showed 13 and 27 significantly associated MHC-class I and II alleles, respectively. The most significant partial haplotype linked with the unsolved CVID was W*01:01:01-DMA*01:01:01-DMB*01:03:01:02-TAP1*01:01:01 ($P < 0.001$), where carriers had a late onset of the disease, only infection clinical phenotype, a non-familial form of CVID, post-germinal center defects and a non-progressive form of their disease. Exclusion of monogenic diseases allowed MR analyses to identify significant genetic variants associated with bacterial infections and improved discrepancies observed in MR analyses of previous GWAS studies with low pleiotropy mainly for a lower respiratory infection, bacterial infection and Streptococcal infection. This is the first study on the full-resolution of minor and major MHC typing and polygenic scores on CVID patients and showed that exclusion of monogenic forms of the disease unraveled an independent role of MHC genes and common genetic variants in the pathogenesis of CVID.

Keywords: primary immunodeficiency, antibody deficiency, common variable immunodeficiency (CVID), whole exome sequencing, full-resolution MHC typing, Mendelian randomization

INTRODUCTION

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID), characterized by impairment of antibody production, recurrent infections and immune dysregulation, in particular, autoimmunity (1). Several different pathogeneses have been suggested from which monogenic diseases cover between 10 and 20% (in Western cohorts) up to 68% (in countries with a high rate of consanguinity) (2–4). Approximately 400 genes have been identified as causative defects of PID, from which half of them have been linked to impaired antibody production and CVID (2). However, additional genetic and non-genetic models have also been considered for CVID (5–7).

Minor and major histocompatibility complex (MHC) genes are the most polymorphic genomic region and specific MHC loci determine the presentation of antigens via B cells to T cells to elicit a germinal center reaction. Physiologically, both MHC class I and II molecules are critical in B cells for stimulating antibody class switching and affinity maturation (MHC class II primarily to follicular helper T cells) and supporting presentation of polysaccharides antigens (mainly via positive signals of MHC class I primarily to natural killer T-cells) (8–10). Given the high prevalence of autoimmune disorders in CVID patients, several studies have investigated the frequency of different MHC alleles in subgroups of CVID patients (11, 12). Furthermore, few reports from different ethnic CVID cohorts have also emphasized a possible contribution of the MHC, mainly class II molecules in patients with gastrointestinal autoimmunity, on the predisposition to CVID (13). In some multiplex families with co-occurrence of CVID and selective immunoglobulin A (IgA), deficiency, MHC markers have also been suggested to play a role both in inheritance and as predictors of progressive disease (14).

However, in CVID patients with a lack of identified monogenic mutations, the disease may occur due to polygenic inheritance involving many common genetic variants with a small effect. An improved methodology for calculating polygenic scores, using a larger cohort of sequenced samples and advanced algorithms, has been proposed to identify a combined impact of single nucleotide polymorphisms (SNP) that significantly increase the risk of disease (15). Mendelian randomization (MR) is an analytical method for identification of causality using polygenic variables and it has been successfully implemented for diseases conferring both monogenic and polygenic traits (16).

Hence we compared the MHC markers and polygenic predictors of CVID patients without a molecular genetic diagnosis after whole exome sequencing (WES), where computational analysis based on high-resolution MHC typing from WES data was performed for the first time. Multi-SNP MR analyses using summary-level data from WES were performed to cross-validate the causality of currently identified variants and previously suggested SNPs for antibody deficiency.

MATERIALS AND METHODS

Study Design and Participants

Patients with a diagnosis of CVID based on the updated clinical diagnostic criteria of the European Society for Immunodeficiencies (ESID, https://esid.org/Working-Parties/Registry-Working-Party/Diagnosis-criteria) and the American Academy of Allergy, Asthma & Immunology (AAAAI) practice parameter for the diagnosis and management of PID (17), were recruited from a cohort of antibody deficiency patients evaluated by WES (2). This cohort was designed to investigate the contribution of genetic, immunologic, and clinical factors of the disease.

Among all registered CVID patients in the Iranian national PID registry (18, 19), available individuals who were referred to the Children's Medical Center (Pediatrics Center of Excellence affiliated to Tehran University of Medical Sciences, Tehran, Iran) and completed the molecular diagnostic investigation were consecutively recruited into this study. Written informed consent for the performed evaluations was obtained from all patients and/or their parents, according to the principles of the ethics committee of the Tehran University of Medical Sciences. An evaluation document was used to summarize the demographic information of the patients, including gender, date of birth, clinical parameters and previous medical history, family history, laboratory and molecular data. A computerized database program (new registry section at, http://rcid.tums.ac.ir/) was designed for the final data collection and direct generation for statistical analysis of data.

Systematic Phenotyping and Genotyping

All clinically diagnosed CVID patients were re-evaluated for fulfilling either the probable or possible diagnostic criteria, and secondary causes of antibody deficiency were ruled out. Clinical phenotyping was performed using a standard method of phenotype subdivision which has been shown to correlate with the quality of life and morbidity among patients with infections only, autoimmunity, lymphoproliferation and enteropathy (20). Based on the epidemiologic data of CVID cohorts worldwide, there are two peak ages of onset, one before the age of ten and another in the third decade of life. Therefore, we defined the early onset as disease onset before age 10 years (20).

Complete blood count, lymphocyte subpopulations, serum Ig levels, and specific antibody responses were measured as previously described (2). Immunological tests were repeated for each patient every 6-months within during routine follow-up visits after the time of diagnosis to evaluate the progression of their antibody deficiency. Patients were classified immunologically based on the main classification for B-cell subsets known as B-cell pattern classification with relevance to genetic findings (21).

Regarding genetic diagnosis, WES was performed according to the protocol described previously (22). For analysis of WES, we followed a published pipeline for prioritizing candidate variants, predicting their effect on protein, homozygosity mapping, large deletion and copy number variation (CNV) detection (2, 22). To classify a patient as a monogenic disease, the pathogenicity of the disease attributable gene variant was re-evaluated using the updated guideline for interpretation of molecular sequencing by the American College of Medical Genetics and Genomics (ACMG), considering the allele frequency in the relevant population database, computational data, immunological/multiomics functional data, familial segregation,

parental data and clinical phenotyping (23). In the remaining patients not only CVID associated genes, but also all known other PID genes were normal (2), therefore we labeled them as "without known genetic mutation detected or unsolved" even though there is a possibility of yet unknown inherited disorder in a minority of them.

MHC Typing Algorithm and Confirmation

The WES data were used for MHC typing of both monogenic and unsolved CVID patients using the major module of Optitype (24) [>97% accuracy (25)]. This algorithm was run according to instructions using fastq files after filtering low-quality reads (base quality of <20 for more than 80% of bases) as an input. In brief, the input data were mapped to the hg38 human reference assembly, and relevant MHC reads from the Binary Alignment Map (BAM) file (chromosome 6, position 29,886,751–33,090,696) were filtered according to their quality scores. Subsequently, four-digit typing, zygosity status and full-resolution imputation were computed. Multiple predictions for an allele at a locus detected in MHC reporter were considered as ambiguous results, and only the first field information was used (26). Confirmatory PCR amplicon-based high-resolution typing was performed on the genomic DNA of the patients as described previously (14, 27).

Polygenic Model and Mendelian Randomization

Exome-wide association study has previously been conducted on 535,486 SNPs extracted from high-throughput sequencing data of both monogenic and unsolved CVID patients as well as 141,456 individuals at Genome Aggregation Database (gnomAD) and 2,497 individuals at Greater Middle East (GME) Variome Project. All SNPs were mapped to build relevant coordinates using liftOver. Prior to imputation, we removed variants with a genotyping rate <98%, ambiguitious SNPs, evidence of deviation from Hardy-Weinberg equilibrium in controls ($p < 1 \times 10^{-4}$), and minor allele frequency $< 1 \times 10^{-6}$. We conducted χ^2 tests of association on genotypes for each cohort separately, using only variants that overlapped between patients cohort and controls. We subsequently only included in the analysis the near-independent SNPs that do not account for linkage disequilibrium (LD) and were significantly different between monogenic and unsolved patients for ease of directly comparing the results.

MR analysis was performed using the identified significant genetic variants, in order to evaluate the effect of exclusion of monogenic patients for prediction of independent common variants without confounding factors, as instrumental variables (serum Ig level) to test for causality (bacterial infections). The result of the MR model on current predictor SNPs of unsolved CVID patients was empowered by comparison of multiple genetic variants reported on previously independent studies on antibody levels using the genome-wide association (GWAS) catalog provided by the National Human Genome Research Institute (NHGRI) and the European Bioinformatics Institute (EMBL-EBI, https://www.ebi.ac.uk/gwas/). Selection of GWAS catalogs on the infectious outcomes were performed to test the causality influenced by the exposures, including ICD10 codes of: J22 Unspecified acute lower respiratory infection (UKB-a:540, $n = 337{,}199$ individuals), A49.9 Bacterial infections of unspecified site (UKB-b:1605, $n = 463{,}010$), A49.8 Other bacterial infections of unspecified site (UKB-b:1399, $n = 463{,}010$), A49.0 Staphylococcal infection, unspecified (UKB-b:3266, $n = 463{,}010$), 0410 Streptococcus infection (UKB-b:4251, $n = 463{,}010$) and A49.1 Streptococcal infection, unspecified (UKB-b:4884, $n = 463{,}010$). Recruitment of GWAS catalogs were performed in the MR-base analytical platform established by the MRC Integrative Epidemiology Unit (University of Bristol, http://app.mrbase.org).

Statistical Approach

Statistical analysis was performed using SPSS (version 21.0.0, SPSS, Chicago, Illinois) and R statistical systems (version 3.4.1.; R Foundation for Statistical Computing, Vienna, Austria) software to compare clinical and immunological parameters between patients with an identified genetic defect and patients with no genetic diagnosis. The one-sample Kolmogorov-Smirnov test was applied to estimate whether data distribution was normal. Parametric and non-parametric analyses were performed based on the finding of this evaluation. Regarding MR, we used the proxy SNPs method instead of LD tagging with minimum LD values of 0.8 and minor allele frequency of (MAF) threshold of aligning palindromes as 0.3. Several MR methods with different sensitivities were applied including Wald ratio, MR Egger, weighted median, and inverse variance weighted algorithms. Forest plot and funnel plot were used to illustrate causality effects and horizontal pleiotropy, respectively. A P-value of $<5 \times 10^{-8}$ was considered for multiple testing and selection of significant SNPs and P-value <0.05 was assumed for comparisons of monogenic and unsolved CVID patients as statistically significant.

RESULTS

Among all genetically evaluated CVID patients, 83 patients agreed to participate in this study (**Table 1**) and monogenic defects were found and confirmed in 40 individuals (2). The remaining 43 "idiopathic" CVID patients were labeled as an unsolved patient. The studied patients (46 males, 37 females) from 71 unrelated kindreds were mainly children and adolescents at the time of the study (43 patients were <18 years old) and parental consanguinity was recorded in 64 patients. The median age of the patients at the onset of symptoms was 3 years (range 0.5–36 years; early-onset manifestation in 79.5%) and the median diagnostic delay (the gap between the onset of the symptoms and diagnosis of CVID) was 4 years (range 0.4–39 years). Of note, 11 patients were from multiplex families (classified as familial cases, 36% with an unsolved disease) and 7 cases progressed to CVID from another form of antibody deficiency during the course of the disease (IgA deficiency and IgG subclass deficiency, 14.2% with an unsolved disease). A summary of the clinical and immunologic phenotype of the studied patients is provided in **Table 1**. There was a significant difference among patients with or without monogenic disorders regarding the age of onset and

TABLE 1 | Clinical and immunologic phenotypes of the 83 CVID patients included in the study.

Parameters	Total CVID patients ($n = 83$)	Monogenic patients ($n = 40$)	Unsolved patients ($n = 43$)	*P*-value
Gender (M/F)	46/37	20/20	26/17	0.16
Median current age, year (range)	18 (5–44)	16 (5–26)	21 (6–44)	0.08
Median age of onset, year (range)	2 (0.5–36)	1.0 (0.5–10)	4.5 (3–36)	0.04*
Median age of diagnosis, year (range)	8 (0.5–30)	7 (0.5–20)	10 (1–30)	0.09
Parental consanguinity (%)	64 (77.1)	31(77.5)	33(76.7)	0.46
Familial patients (%)	11 (13.2)	7 (17.5)	4 (9.3)	0.13
CLINICAL PHENOTYPE				
Infections only (%)	25 (30.1)	9 (22.5)	16 (37.2)	0.07
Autoimmunity (%)	29 (34.9)	16 (40.0)	13 (30.2)	0.17
Lymphoproliferation (%)	30 (36.1)	16 (40.0)	14 (32.5)	0.24
Enteropathy (%)	23 (27.7)	11 (27.5)	12 (27.9)	0.48
Malignancy (%)	4 (4.8)	3 (7.5)	1 (2.3)	0.13
Allergy (%)	10 (12.0)	7 (17.5)	3 (6.9)	0.07
Overlap phenotype (%)***	31 (37.3)	18 (45)	13 (30.2)	0.08
IMMUNOLOGIC PHENOTYPE				
Progressive form of antibody deficiency (%)	7 (8.4)	6 (15)	1 (2.3)	0.01*
White blood cells/ul (SD)	8,051.5 (3,504.2)	7,955.8 (2,119.0)	8,257.2 (2,738.9)	0.43
Lymphocytes/ul (SD)	2,890.5 (1,024.9)	2,784.0 (1,935.4)	3,137.2 (2,344.0)	0.31
B cells, % (SD)	10.5 (7.8)	10.2 (4.4)	11.3 (5.9)	0.49
IgM, mg/dl (SD)	18.7 (10.2)	17.3 (7.5)	21.1 (6.8)	0.24
IgG, mg/dl (SD)**	275.9 (251.3)	337.8 (229.1)	241.0 (142.8)	0.09
IgA, mg/dl (SD)	22.8 (12.0)	27.8 (11.9)	13.9 (10.6)	0.07
B CELL SUBSET PHENOTYPE				
Pattern 1 (low transitional and memory B cells) (%)	23 (27.7)	8 (20.0)	10 (23.2)	0.35
Pattern 2 (low naïve mature, marginal zone-like and memory B cells) (%)	6 (7.2)	3 (7.5)	3 (6.9)	0.46
Pattern 3 (low marginal zone-like and memory B cells) (%)	11 (13.2)	7 (17.5)	4 (9.3)	0.13
Pattern 4 (low memory B cells) (%)	26 (31.3)	15 (37.5)	11 (25.5)	0.12
Pattern 5 (post-germinal center defect) (%)	17 (20.4)	7 (17.5)	15 (34.8)	0.04*

*Statistically significant difference, $p < 0.05$.

**Values at the time of CVID diagnosis and before immunoglobulin substitution.

***Overlap phenotype: CVID patients that develop more than one non-infectious complications and present with at least two concurrent complications of autoimmunity, lymphoproliferation, and enteropathy (2, 20).

progressive form of CVID, while parental consanguinity and familial cases were comparable.

We first investigated the frequency of MHC class I and II alleles in the CVID patients. Among the 83 patients, high-resolution WES-based typing of class I revealed the highest diversity in MHC-B with 84 unique alleles (mainly B*35, 31 alleles out of total 166 alleles:18.6%). However, the CVID cohort had a restricted MHC-H repertoire with only 4 unique alleles (mainly H*02, 113 alleles: 68.0%, **Tables S1–S8**). Evaluation of MHC class II showed that the most diverse locus was MHC-DPB1 with 40 unique alleles (mainly DPB1*463:01:01, 23 alleles: 13.8%), in contrast to three unique alleles for MHC-DRB4 (mainly DRB4*01:03:01, 135 alleles: 81.3%, **Tables S9–S23**). The most significant increases in the proportions of class I in the unsolved patient cohort were observed in B*39 ($p = 0.02$), B*50:01:01:01 ($p = 0.02$), and E*01:08N ($p = 0.02$, **Table 2**, **Figures 1A,B**). Moreover, susceptibility class II regions for unsolved CVID were most significantly associated with DQA1*01:04:01 ($p < 0.001$), DQB1*03:01:01 ($p = 0.002$), DPA1*01:03:01:04 ($p = 0.002$), and TAP1*01:01:01:01($p = 0.002$, **Table 3**, **Figures 1A,C**). There were no significant differences in the frequency of alleles of MHC–H, –G –DRB3, and –DRB4 between monogenic and unsolved CVID patients (**Tables S6, S7, S20, S21**, **Figure 1**).

Regression model analysis using the identified significant MHC alleles suggested a combination of B*35, DMA*01:02, TAP1*06:01, MICB*002:01, DQA1*01:04:01, and DQB1*03:01:01 as the best fit model to predict an unsolved form of CVID ($p = 4.5 \times 10^{-6}$, **Table S24**). In the second model, we tested for an association with a significant haplotype in patients with unsolved CVID. In this model, the W*01:01:01-DMA*01:01:01-DMB*01:03:01:02-TAP1*01:01:01 ($p < 0.001$), was the most significant associated haplotype with an increased unsolved CVID odds (**Table 4**). This haplotype was exclusively

TABLE 2 | Significantly different MHC-class I alleles associated with monogenic and unsolved CVID patients.

MHC class I	Monogenic patients (n = 40/alleles = 80)	Homozygous monogenic patients	Unsolved patients (n = 43/alleles = 86)	Homozygous unsolved patients	OR	Effect on unsolved CVID	*P*-value
A*02:05:01:01	4	0	0	0	NI	P	0.017*
A*24	18	2	10	1	1.93	P	0.03*
A*24:02:01:01	12	0	5	0	2.58	P	0.02*
A*33	1	0	6	0	0.17	S	0.03*
A*33:03:01	0	0	3	0	NI	S	0.04*
A*68:01:01:02	4	0	0	0	NI	P	0.017*
A*68:02:01:01	0	0	3	0	NI	S	0.04*
B*07:02	0	0	3	0	NI	S	0.04*
B*35	11	2	20	4	0.59	S	0.05*
B*35:03	1	0	6	1	0.17	S	0.03*
B*35:03:19	0	0	3	0	NI	S	0.04*
B*35:08:01:01	0	0	3	0	NI	S	0.04*
B*38	6	0	2	0	3.22	P	0.05*
B*38:60	4	0	0	0	NI	P	0.017*
B*39	0	0	4	0	NI	S	0.02*
B*50:01:01:01	0	0	4	1	NI	S	0.02*
B*50:01:01:02	4	0	0	0	NI	P	0.017*
B*58	3	0	0	0	NI	P	0.03*
C*04:01:01:03	0	0	3	0	NI	S	0.04*
C*04:243	0	0	3	1	NI	S	0.04*
C*07:01:01:16	3	1	0	0	NI	P	0.03*
E*01:01:01:01	9	3	3	0	3.22	P	0.02*
E*01:01:01:04	9	1	19	5	0.50	S	0.03*
E*01:03:01:03	8	2	3	1	2.86	P	0.04*
E*01:08N	0	0	4	1	NI	S	0.02*
F*01:01:01:01	13	2	7	0	1.99	P	0.05*
F*01:01:02:04	3	0	9	2	0.35	S	0.04*
W*01:01:01	43	4	58	2	0.79	S	0.03*

OR, odds ratio; NI, not calculable; P, preventive for unsolved CVID; S, susceptibility for unsolved CVID.
**Statistically significant difference, $p < 0.05$.*

identified in 11 patients without a genetic diagnosis. The majority of these patients had a late onset ($n = 9$, 81.8%) and none of them were of a familial CVID or progressive form of the disease. Infections only phenotype ($n = 10$, 90.9%) and post-germinal center defects ($n = 8$, 72.7%) were the main clinical and immunologic phenotypes in these patients.

Exome-wide significant results between monogenic and unsolved CVID patients and variants with the strongest association compared to healthy individuals were selected for MR analysis. The distribution of significant variants supported a polygenic etiology of unsolved CVID (**Figure 2A**). Correlation matrix and principal component analysis (PCA) of selected variants showed two distinct sets of SNPs, discriminating monogenic and unsolved CVID patients (**Figures 2B,C**). Subsequently, the selected variants were incorporated into a MR model considering them as directly linked to antibody deficiency (**Figure 2D**) and compared with previously suggested variants found in GWAS studies of antibody deficient patients without a genetic evaluation (**Table S25**). **Table 5** and **Table S26** summarize the MR estimates from each method of the causal effect of the exposures (variants of the current study and previous GWAS studies) on the susceptibility to infectious diseases as an outcome. The Wald ratio effects reported in the previous GWAS studies, neglecting the exclusion of monogenic diseases, showed only a significant negative correlation of IgG levels on streptococcal infection ($p = 0.002$) and IgA levels on bacterial infection ($p = 0.04$). Although our MR analysis approach also only provide a link between unsolved CVID with bacterial infections, all the estimated coefficients for other infectious diseases were directly associated with these SNPs, whereas several discrepancies were have been observed in MR analyses of previous GWAS studies (**Table S26**).

To evaluate the consistency of the causal estimate of all SNPs observed, the variability in the estimates obtained for each SNP was calculated and heterogeneity was only significant in association with IgA level and lower respiratory infections in previous GWAS studies (MR Egger, $p = 0.0089$ and inverse variance weighted, $p = 0.016$, **Table S27**) but none in our suggested model (**Table S28**). Forest plots showed that the

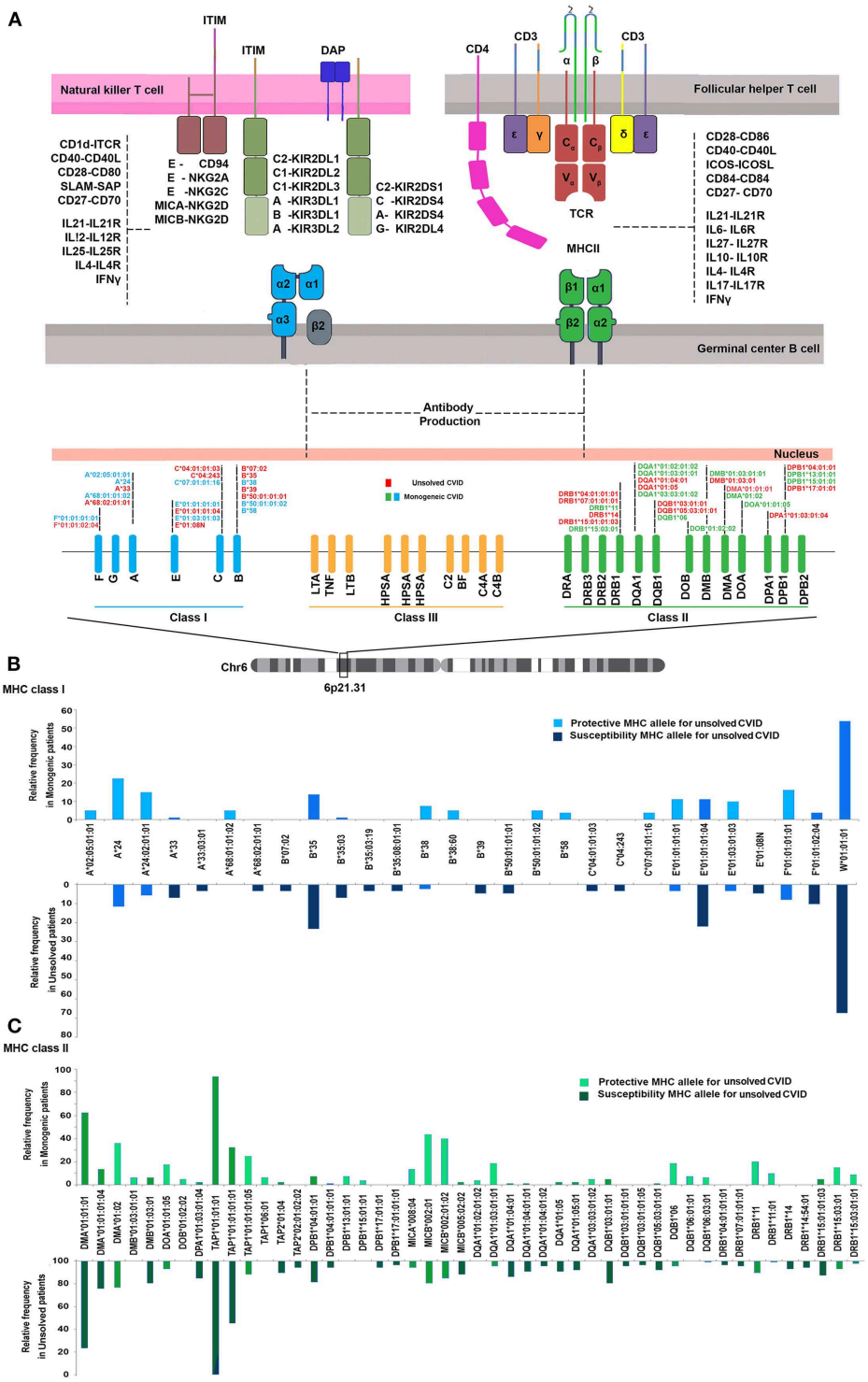

FIGURE 1 | (A) Schematic presentation of association of MHC status in the pathogenesis of common variable immunodeficiency based on the role of MHC in antigen presentation within germinal center B cells. **(B)** MHC class I **(C)** and MHC class II risk and protective alleles for unsolved CVID compared to monogenic-CVID patients.

causal effect of every single SNP in previously published GWAS studies had a non-unified prediction on outcome in line with formal MR estimates of heterogeneity as shown in **Figures S1, S2**. However, the symmetric influence of SNPs selected by the exclusion of monogenic CVID on the outcome was observed in our dataset for all infectious

TABLE 3 | Significantly different MHC-class II alleles associated with monogenic and non-monogenic CVID patients.

MHC class II	Monogenic patients (n = 40/alleles = 80)	Homozygous monogenic patients	Unsolved patients (n = 43/alleles=86)	Homozygous unsolved patients	OR	Effect on unsolved CVID	*P*-value
DMA*01:01:01	50	21	66	29	0.81	S	0.02*
DMA*01:01:01:04	11	4	21	8	0.56	S	0.04*
DMA*01:02	29	10	20	6	1.55	P	0.03*
DMB*01:03:01:01	5	2	0	0	NI	P	0.009**
DMB*01:03:01	5	0	17	0	0.31	S	0.005**
DOA*01:01:05	14	6	6	2	2.50	P	0.01*
DOB*01:02:02	4	1	0	0	NI	P	0.01*
DPA1*01:03:01:04	2	0	13	2	0.16	S	0.002**
TAP1*01:01:01	75	34	86	42	0.94	S	0.009**
TAP1*01:01:01:01	26	12	47	16	0.60	S	0.002**
TAP1*01:01:01:05	20	6	10	3	2.15	P	0.01*
TAP1*06:01	5	1	0	0	NI	P	0.009**
TAP2*01:04	2	0	9	3	0.23	S	0.01*
TAP2*02:01:02:02	0	0	5	2	NI	S	0.01*
DPB1*04:01:01	6	1	16	2	0.40	S	0.01*
DPB1*04:01:01:01	1	0	5	1	0.21	S	0.03*
DPB1*13:01:01	6	1	0	0	NI	P	0.004**
DPB1*15:01:01	3	1	0	0	NI	P	0.03*
DPB1*17:01:01	0	0	5	0	NI	S	0.01**
DPB1*17:01:01:01	0	0	3	0	NI	S	0.04*
MICA*008:04	11	5	5	2	2.36	P	0.04*
MICB*002:01	35	16	17	6	2.21	P	<0.001***
MICB*002:01:02	32	15	13	6	2.64	P	<0.001***
MICB*005:02:02	2	0	10	0	0.21	S	0.01*
DQA1*01:02:01:02	3	1	0	0	NI	P	0.03*
DQA1*01:03:01:01	15	6	4	1	4.03	P	0.002**
DQA1*01:04:01	1	0	12	3	0.08	S	<0.001***
DQA1*01:04:01:01	1	0	8	2	0.13	S	0.01*
DQA1*01:04:01:02	0	0	4	1	NI	S	0.02*
DQA1*01:05	2	0	8	1	0.26	S	0.03*
DQA1*01:05:01	2	0	7	1	0.30	S	0.05*
DQA1*03:03:01:02	4	2	0	0	NI	P	0.01*
DQB1*03:01:01	4	1	17	4	0.25	S	0.002**
DQB1*03:01:01:01	0	0	4	0	NI	S	0.02*
DQB1*03:01:01:05	0	0	3	1	NI	S	0.04*
DQB1*05:03:01:01	1	0	7	0	0.15	S	0.01*
DQB1*06	15	4	4	0	4.03	P	0.004**
DQB1*06:01:01	6	3	0	0	NI	P	0.004**
DQB1*06:03:01	5	1	1	0	5.37	P	0.03*
DRB1*04:01:01:01	0	0	3	0	NI	S	0.04*
DRB1*07:01:01:01	0	0	4	0	NI	S	0.02*
DRB1*11	16	6	9	1	1.91	P	0.04*
DRB1*11:01	8	3	1	0	8.6	P	0.006**
DRB1*14	0	0	6	2	NI	S	0.008**
DRB1*14:54:01	0	0	5	2	NI	S	0.01**
DRB1*15:01:01:03	4	0	11	0	0.39	S	0.04*
DRB1*15:03:01	12	4	6	0	2.15	P	0.04*
DRB1*15:03:01:01	7	3	2	0	3.76	P	0.03*

OR, odds ratio; NI, not calculable; P, preventive for unsolved; CVID, S, susceptibility for unsolved CVID.
**Statistically significant difference, $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

diseases evaluated, except unspecific bacterial infections (**Figure 3**). Asymmetry and larger spread of β_{IV} in the Funnal plots also suggests a higher heterogeneity and presence of horizontal pleiotropy due to the absence of exclusion of monogenic disease in previous GWAS studies (**Figures S3, S4**), whereas a homogenous β_{IV} value was observed for lower respiratory infection, bacterial infection, and Streptococcal infection in currently identified SNPs (**Figure S5**). Of note, the polygenic score of unsolved CVID disease was increased when the testing dataset had a lower percentage of MHC risk factor (**Figure 2E**).

DISCUSSION

In this study, we demonstrated the importance of exclusion of monogenic CVID on the evaluation of the effects of MHC alleles and polygenic allele scores in MR analyses in the remaining patients. Since the late 1970s, when the association of MHC locus was initially determined as a principal genetic factor in antibody deficient patients (28, 29), the debate still is ongoing about the direct (the presentation of antigens by MHC) or indirect (association with the adjacent gene within the same region) role of these markers on the pathogenesis of these diseases. However, recently, several monogenic diseases originating from genes outside chromosome 6, the genomic location of MHC, have been discovered to underlie these disorders (2–4, 30–33). For the first time, we used this patient group as a control which may deconfound the effect of other genetic etiologies and may identify the difference in MHC markers between patients with and without monogenic defects. Moreover, using high-resolution MHC typing we also investigated both minor and major histocompatibility complex genes which have not been performed hitherto.

The distribution of major histocompatibility alleles in the current study, compared to our previous investigation on a mixed CVID patient population using low-throughput PCR-based molecular DNA typing, provides reproducible data on MHC class II (with proportional increase in DQB1*0201, DQA1*0103, and DRB1*15 alleles) (11), indicating the advantage of next-generation sequencing by generating data on a larger patients sample with a high resolution of the MHC typing. The findings of the current investigation also conferred that despite some observed significant differences between mixed CVID populations and healthy controls (DRB1*4, DRB1*11, DRB1*07), the design of previous studies has led to false positivity in six alleles and false negativity in 14 alleles associated with MHC class II of unsolved CVID (11). Therefore, subtracting the monogenic patients is an essential factor that should be considered when evaluating whether inheritance of a particular MHC haplotype is associated with CVID development.

In a study designed by Waldrep et al. (12) to test for the possibility of synergy (epistasis) between a mutant transmembrane activator and calcium-modulator and cyclophilin-ligand interactor (TACI) and genes located near the MHC class I locus, they stratified patients based on the variants identified in the *TNFRSF13B* gene. Although the strength of the

TABLE 4 | Distribution of MHC haplotypes among unsolved CVID patients vs. monogenic CVID patients (significant haplotypes with frequency ≥5% are shown).

MHC-B	MHC-E	MHC-W	MHC-DMA	MHC-DMB	MHC-TAP1	MHC-DPB1	MHC-DQB1	Monogenic patients (alleles = 80)	Unsolved patients (alleles = 86)	OR	*P*-value
B*35		W*01:01:01	DMA*01:01:01		TAP1*01:01:01			6	16	0.40	0.01*
B*35		W*01:01:01	DMA*01:01:01	DMB*01:03:01:02	TAP1*01:01:01			0	7	NI	0.003**
B*35							DQB1*03:01:01	2	9	0.23	0.01*
B*35						DPB1*04:01:01		1	7	0.15	0.01*
B*35				DMB*01:03:01:02				0	7	NI	0.003**
	E*01:01:01:04	W*01:01:01	DMA*01:01:01		TAP1*01:01:01			2	9	0.23	0.01*
	E*01:01:01:04					DPB1*04:01:01		0	8	NI	0.002**
		W*01:01:01	DMA*01:01:01	DMB*01:03:01:02	TAP1*01:01:01			0	11	NI	<0.001***
		W*01:01:01	DMA*01:01:01		TAP1*01:01:01	DPB1*04:01:01		0	9	NI	0.001**
		W*01:01:01	DMA*01:01:01		TAP1*01:01:01	DPB1*04:01:01	DQB1*03:01:01	0	6	NI	0.007**
		W*01:01:01	DMA*01:01:01		TAP1*01:01:01		DQB1*03:01:01	2	10	0.21	0.007**
						DPB1*04:01:01	DQB1*03:01:01	0	6	NI	0.007**

OR, odds ratio; NI, not calculable.

**Statistically significant difference, $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

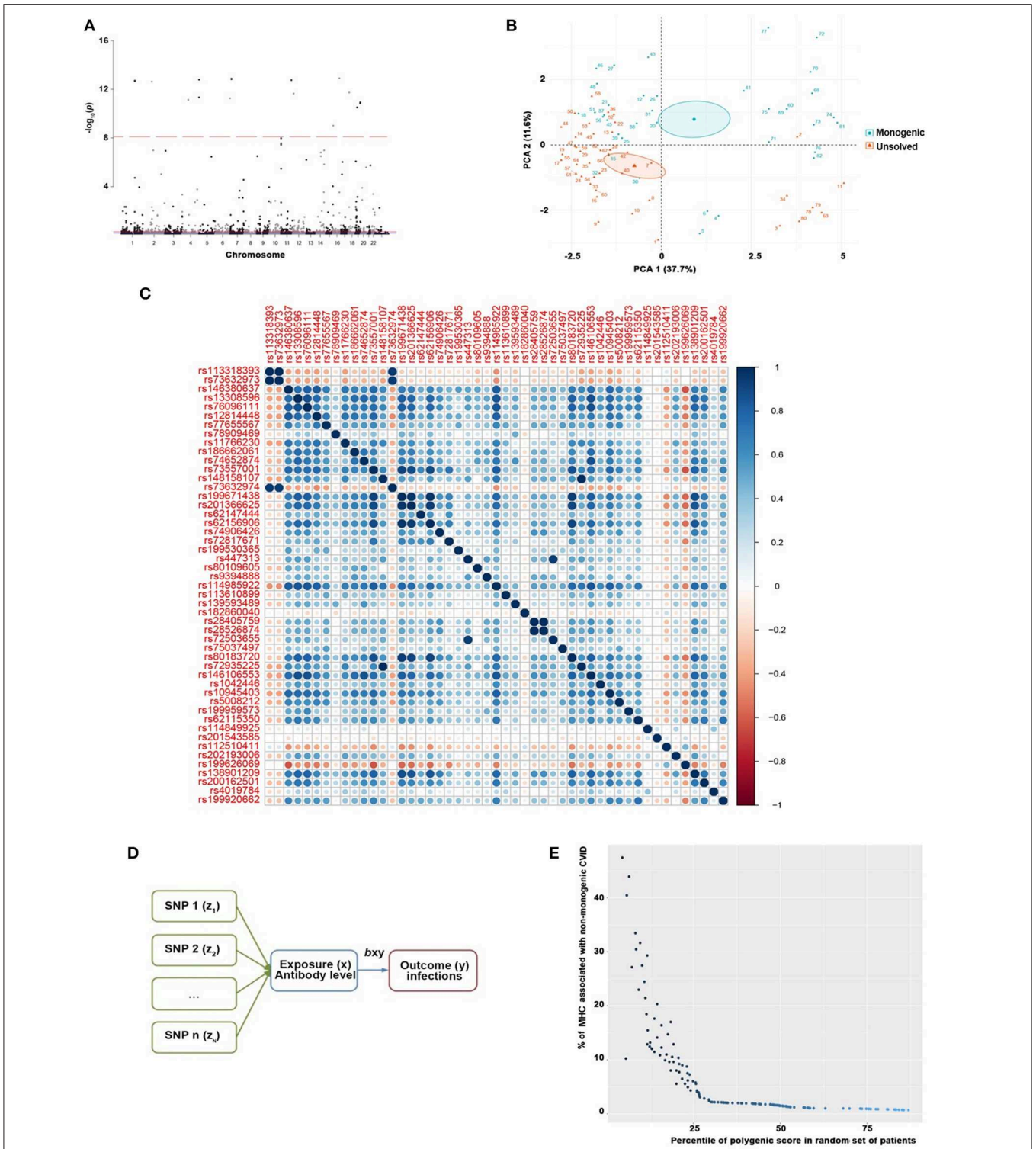

FIGURE 2 | (A) Manhattan plot of exome-wide significant results of 535,486 SNPs between monogenic and unsolved CVID patients supporting a polygenic etiology of unsolved CVID where variants with a genotyping rate <98%, ambiguitious SNPs, evidence of deviation from Hardy-Weinberg equilibrium in controls ($p < 1 \times 10^{-4}$), and minor allele frequency $< 1 \times 10^{-6}$ were removed. Only the near-independent SNPs that do not account for linkage disequilibrium and were significantly different between monogenic and unsolved patients were included for the next step. **(B)** Principal component analysis (PCA) and **(C)** correlation matrix of significant variants discriminating monogenic and non-monogenic CVID patients. **(D)** MR model generated for selected variants were inputed to a considering them as direct linked with exposure (antibody deficiency) and outcome (infectious diseases). **(E)** Polygenic score of unsolved CVID disease was increased when the testing dataset had a lower percentage of MHC risk alleles.

TABLE 5 | MR estimates from each method of the causal effect of the exposures (variants of current studies and GWAS studies) on the infectious diseases as outcome.

Outcomes	MR method	β	Standard error	*P*-value
Lower respiratory infection	MR Egger	0.002238	0.002275	0.358
	Weighted median	0.0001442	0.0001085	0.1838
	Inverse variance weighted	0.00009826	0.00007786	0.2069
	Weighted mode	0.0001789	0.000178	0.3442
Staphylococcal infection	MR Egger	0.00001533	0.0002204	0.9465
	Weighted median	−0.000003872	0.000007605	0.6106
	Inverse variance weighted	−0.000001176	0.00000711	0.8686
	Weighted mode	−0.000003908	0.00001376	0.7835
Bacterial infection	MR Egger	0.002014	0.0001429	0.02016*
	Weighted median	0.00007075	0.000006549	0.028*
	Inverse variance weighted	0.00008677	0.000004927	0.04821*
	Weighted mode	0.00007561	0.0000103	0.04838*
Other bacterial infections	MR Egger	−0.00005373	0.0001598	0.7465
	Weighted median	0.000001372	0.000007065	0.8461
	Inverse variance weighted	−6.593e-7	0.000005509	0.9047
	Weighted mode	0.000004278	0.00001016	0.6848
Streptococcus infection	MR Egger	0.000005027	0.0002213	0.9825
	Weighted median	−0.000005359	0.000007243	0.4594
	Inverse variance weighted	−0.000002277	0.000007138	0.7498
	Weighted mode	−0.000004368	0.00001126	0.7082
Streptococcal infection, unspecific	MR Egger	0.00009312	0.0001238	0.4764
	Weighted median	0.000002508	0.000005664	0.6579
	Inverse variance weighted	0.000002418	0.000004267	0.571
	Weighted mode	0.000002594	0.000008395	0.7652

study was not sufficient and only evaluated the polymorphic region of the *TNFRSF13B* gene (exons 3 and 4), their preliminary data support the hypothesis that the overall pattern of MHC alleles in individuals with a mutated TACI allele is different than in individuals with idiopathic CVID. This notion is consistent with the observation in our study by excluding all monogenic disorders underlying CVID and evaluation of both MHC class I and II alleles.

Most of the previous works on genetic susceptibility factors in CVID patients have only focused on MHC class II due to its known role in antibody class switching and affinity maturation. Using a limited number of CVID patients, we showed previously that MHC haplotypes, including DRB1*04-DQB1*03:01-DQA1*03:01 and DRB1*01:01-DQB1*03:01-DQA1*05:05 confer susceptibility to CVID, while DRB1*07-DQA1*02:01 constitutes a protective haplotype (11). A restricted diversity of MHC class II, in particular, MHC-DR, has also been reported previously in patients with a familial form of CVID with first degree relatives showing IgA deficiency (DRB1*03:01-DQB1*02:01 and DRB1*04) (34–36).

Both MHC class I and II markers in CVID patients could predict the clinical presentation and immunologic profile including enteropathy and autoimmunity (DQ*02:05 and DQ*8) (13), chronic inflammation (A*29) (37), severe infectious complication (A*11 and B*44) (38, 39), progressive disease (A*24, DQB1*03:01 and DQA1*05:01) (40, 41), and number of marginal zone-like B cells and switched memory B cells (B*8 and B*44) (42). However, MHC class I and other minor histocompatibility variants in this region have not been evaluated in most previous CVID studies. In a few reports, the effect of an increased proportion of A*24, B*14, A*02-B*40, A*02-B*044, A*03-B*07, A*01-B*08:01, and B*44-C*16, and a reduced proportion of B*62 and C*7 on the function of NK cells in CVID patients have been demonstrated (43–46). One previous study has also suggested that the CVID risk is increased in patients where Killer cell immunoglobulin-like receptors (KIR)/MHC class I combinations facilitate NK cell activation (B*44-KIR3DS1 and C*16-KIR2DL3) (46).

With a full resolution MHC typing, we identified a hitherto not recognized, novel MHC haplotype (W*01:01:01-DMA*01:01:01-DMB*01:03:01:02-TAP1*01:01:01), which is associated with unsolved CVID in patients with a late onset of symptoms, present a non-progressive form of the disease with an infections only phenotype and post-germinal center defects. Of note, the proportion of some specific MHC markers was also increased in the monogenic CVID patients which could suggest the deprivation of non-monogenic patients from those MHC alleles or it may be due to linkage of MHC markers with monogenic disorders. However, the latter is less likely since the 40 patients included in this study with the monogenic disease have 26 different genetic problems (2).

We also performed a simulation study on common genetic variants with significant differences between monogenic and unsolved CVID patients as an instrument in MR and compared them to previous GWAS studies. The data supports the notion that variants included in our model satisfy the assumptions of Ig production and bacterial infections as instrumental variables. Since the observed SNPs were consistent for prediction of infectious diseases, the cohort sample bias was not significant. Although the effect of sample size and usage of these unweighted allele scores may influence the predictivity and significance of the genetic model, where exclusion of monogenic forms of CVID unified the effect direction on all evaluated outcomes. Although

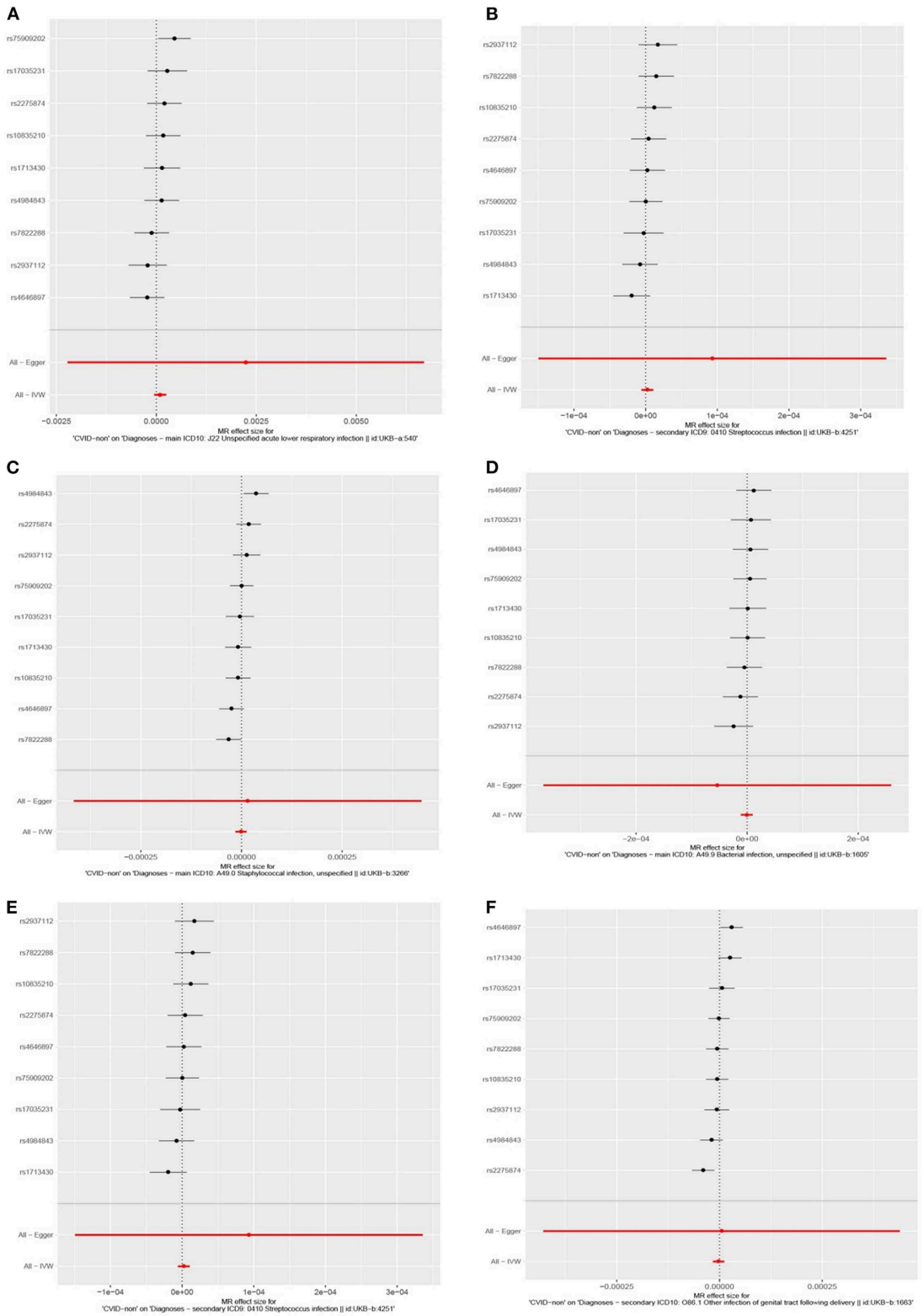

FIGURE 3 | Forest plots depicting causal effect of every single SNP in this cohort by exclusion of monogenic CVID patients suggesting a unified prediction on outcome for all infectious diseases including **(A)** lower respiratory infection, **(B)** staphylococcal infection, **(C)** bacterial infections, **(E)** Streptococcus infection, **(F)** Streptococcal infection, unspecific, except unspecific bacterial infections **(D)**.

imprecisely weighted allele scores would not bias our observed estimates, we suggest further cross-validation with other CVID cohorts before integration of weights in the MR data analysis to prevent reduction of power.

Since the number of SNPs used for instruments do not influence the effect size, we have included all significant markers discriminating between monogenic and unsolved CVIDs. It has been previously recommended that variants should be selected on the basis of scientific knowledge rather than statistical testing. However, in a majority of previous MR studies, all variants which can be reasonably assumed to be valid instruments have been considered during analysis to improve the precision of the causal estimate. However, we also performed a pathway analysis for instrumented SNPs representing their function in the immune system, particularly O-glycosylation of proteins and TNFR1-induced proapoptotic signaling, which are important for immunoglobulin production (**Table S29**).

We have also demonstrated that the use of multiple genetic variants in the context of MR has a significant impact on the strength of predictive MHC markers, with slight reductions in power of polygenic scores in CVID carriers of significant MHC markers. These findings support the notion of two separate mechanisms of MHC and polygenic variants in individuals with unsolved CVID. MHC haplotype is a genetic susceptibility factor for CVID which has been identified as a modifying factor for clinical presentation and immunologic phenotype. However, with current findings, we suggest MHC typing and polygenic evaluation of unsolved CVID patients should be integrated into the flowcharts of genetic screening (2). Moreover, with finding population-specific MHC and SNP predictors we can identify the at-risk asymptomatic individuals within the families of CVID patients and follow them up to improve the prognosis of the disease.

Considering the limitation of the current data of using multiple genetic variants, allele scores in particular, and missing data leading to reduced sample sizes for analysis, future studies in multiple variant setting in the whole genomic level and with higher samples size of monogenic and unsolved patients (from a similar population) could help imputation as it has been shown to be effective against any reduction in power due to missing data. The generalization of discovered MHC and SNP markers in unsolved patients should be performed with caution considering the genetic variations of the cohort population (mainly early-onset, higher rate of consanguinity and lower delay in diagnosis may be due to earlier and severe presentation), however, the methodology presented in the current study would be a commonly recommended approach after performing next-generation sequencing. Although familial cases were slightly higher in a group of patients with identified genetic defects, we cannot conclude at least from our data that multiple cases in a family suggest absolutely the monogenic form of CVID, the fact which is consistent with data from several Western cohorts of patients with accumulation of IgAD and CVID in a family without defining underlying genetic defect but similar MHC markers.

Although the exclusion of monogenic disorders in a newly clinically diagnosed CVID patient is a first mandatory step toward evaluation of other pathogenic mechanisms, detection of the causative genomic element is a challenging task in the MHC region in idiopathic patients due to its complexity and density of genes. Based on current data both MHC alleles and their adjacent genes are involved directly or indirectly in the etiology of some of unsolved CVID patients. Therefore, full sequencing of the MHC region in large populations of CVID patients is recommended. For patients with monogenic diseases, MHC typing may also unravel some markers for variation of the clinical presentation in patients with the same mutation or as a predictive marker for morbidity and mortality. However, to prove this we need a global effort to access a significant amount of patients with unique gene defects or better with a unique mutation within a specific gene. Moreover, the current findings indicate the probability of poly-genic etiology in idiopathic CVID patients. However, before extrapolating the polygenic scores observed in these patients, application of instrumental variable methods with genetic instruments to estimate the causal effect of reduction of immunoglobulin levels (rather than other defects in immune or non-immune system) on the infection diseases should be evaluated further with future observational data.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Karolinska Institutet and Tehran University of Medical Sciences. Written informed consent to participate in this study was provided by the participants and/or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HA designed the project, collected the clinical data, interpreted the analysis, and wrote the paper. CL performed the bioinformatic analysis and analyzed and interpreted the data. AA collected the clinical materials and followed up the patients. LH designed the project, analyzed and interpreted the data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00014/full#supplementary-material

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Functions of TFH Cells in Common Variable Immunodeficiency

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Common variable immunodeficiency is the most common clinical primary immunodeficiency in adults. Its hallmarks are hypogammaglobulinemia and compromised B-cell differentiation into memory or antibody-secreting cells leading to recurrent infections. This disease is heterogeneous, with some patients harboring multiple complications such as lymphoproliferative disorders, autoimmune manifestations, or granulomatous inflammation. The mechanisms leading to these complications remain elusive despite numerous associations found in the literature. For instance, although described as a B cell intrinsic disease, numerous abnormalities have been reported in other immune cell compartments. Here, we tuned our attention to follicular helper T cells, a $CD4^+$ T cell population specialized in B cell help, considering the recent publications showing an involvement of these cells in CVID pathogenesis.

Keywords: follicular helper T cells, CVID, complications, B cells, IFNγ

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INTRODUCTION

Common variable immunodeficiency (CVID) is an umbrella name for the most common symptomatic, but also the most heterogeneous, primary antibody deficiency in adults. Typical clinical features of this heterogeneous group of disorders include recurrent infections, decreased serum immunoglobulin (Ig) and impaired specific antibody (Ab) responses to vaccines reflecting impaired B cell responses (1). Diagnosis criteria recently defined by the European Society for ImmunoDeficiencies include at least one of the following: increased susceptibility to infections, autoimmune manifestations, granulomatous disease, unexplained polyclonal lymphoproliferation, or affected family member with antibody deficiency. Moreover, the following parameters should be present to confirm the diagnosis: diagnosis after the age of 4 years, no evidence of profound T-cell deficiency, deficit in serum Ig (multiple classes) not explained by other known causes, and impaired vaccination responses or low switched memory B cells (smB cells) (2, 3). CVID has a complex genetic basis, with monogenetic causative forms and genetic predispositions (4), as reviewed in Cunningham-Rundles (5). Some CVID forms are inherited, but family members of CVID patients are usually normal and not all individuals who inherit a gene mutation associated with CVID will develop the disease (6). Nevertheless, a genetic cause has been identified in about 25% of CVID patients using next-generation sequencing. As examples, mutations in several genes encoding for B cell receptor complex associated proteins, B cell activating factor receptor (BAFF-R), inducible co-stimulator (ICOS), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), phosphatidylinositol 3-kinase (PI3K), and in lipopolysaccharide-responsive beige-like anchor (LRBA) protein or more recently the NFκB family have been described (5–7). Mutations in the *TNFRSF13B* gene encoding

the transmembrane activator and CAML interactor (TACI) are found in 8–10% of patients (8) but relatives to CVID patients with mutations in TACI display normal levels of Ig. The identification of mutations in genes encoding factors important in B cell generation or differentiation is not surprising, as CVID patients present abnormalities in the B cell compartment. In fact, impaired B cell differentiation is a hallmark of the disease and, despite normal levels of total B cells in most cases, post-germinal center (GC) B cells are defective and patients harbor lower levels or absence of smB cells (9, 10). Consequently, multiple CVID classifications based on B-cell phenotype have been proposed. On top of these classifications, two groups of patients are often described in the literature, namely one comprising patients that show only recurrent infections, and the other with patients harboring at least one of the following complications: (i) benign, granulomatous, or malignant lymphoproliferation, (ii) chronic enteropathy, and (iii) autoimmune manifestations. Moreover, a report in 2014 of the largest cohort of CVID patients studied so far highlighted that an early-onset of CVID (before the age of 10) is associated with infections (especially pneumonia) rather than other complications, suggesting two distinct disease entities (11). The pathogenesis leading to immune disorders of CVID is still poorly understood, but functional impairments in multiple immune cell types may be responsible for some of the pathophysiology of CVID.

IMMUNOLOGICAL FEATURES OF CVID PATIENTS WITH NON-INFECTIOUS COMPLICATIONS

More than half of the patients harbor non-infectious complications causing increased morbidity and mortality (12). Cancers occur in 20% of CVID patients, the majority of cancers being lymphoma (13, 14). More than 25% of CVID patients have autoimmune complications (15). Immune thrombocytopenia (ITP) and autoimmune hemolytic anemia are the most frequent disorders, but many others such as vitiligo, pernicious anemia, systemic lupus erythemateous, rheumatoid arthritis, antiphospholipid syndrome, juvenile idiopathic arthritis, Sjögren's disease, psoriasis, thyroiditis, uveitis, and vasculitis can also be found in CVID patients (15). As impairment of B cell maturation is a hallmark of the disease, these cells have drawn a lot of attention. Wehr et al. have shown a significant decrease in isotype-switch memory B cells in patients with non-infectious complications such as autoimmunity, granulomatous disease, lymphoid hyperplasia, or splenomegaly (12). Intriguingly, despite defects in B cell differentiation and serum Ig, CVID patients develop autoantibodies and autoimmune manifestations. Such a paradigm might be due to a default in specific checkpoints for autoreactive B cells, although this hypothesis has yet to be proven. Interestingly, autoimmunity in CVID has been associated with the presence of $CD21^{low}$ B cells, an "innate-like" population expressing low levels of CD38 but exhibiting autoreactivity (16, 17). Moreover, an increase of $CD21^{low}$ B cells has been observed in CVID patients presenting immune thrombocytopenia (ITP) (18). It has been shown that $CD21^{low}$ cells may develop from memory B cells under chronic inflammatory conditions and are present at high levels in autoimmune patients (19). These observations suggest a role for these $CD21^{low}$ smB cells in the development of autoimmune complications observed in CVID patients, but this possibility remains to be explored.

Beyond the impairment of B cell functions, numerous immune alterations have been described in CVID patients with non-infectious manifestations. For instance, dysfunctions in monocytes/macrophages, dendritic cells (20), NK cells and innate lymphoid cells (ILCs) have been reported. Monocytes have impaired antigen-presenting capacities but increased capacity to produce reactive oxygen species or IL-12 (21). By contrast, IL-12 production by dendritic cells from CVID patients is lower than that of healthy donors, reflecting a defective maturation of these cells (22, 23). Two studies have reported a decrease in ILCs, either in $CD127^{+}CD90^{+}$ ILCs (24) or in ILC2s (25). By contrast, a study from Cols et al. (26) shows an expanded population of ILCs harboring an IFNγ signature in patients with non-infectious complications, suggesting that ILCs may be a critical source of IFNγ in these patients. Overall, defining the roles of ILCs in CVID pathogenesis still needs further investigation.

Numerous studies have reported abnormalities in the T-cell compartment [as reviewed in (27)], which is not surprising given the central role of T cells, especially CD4 T cells, in B cell activation and differentiation into memory and Ig-producing cells. Patients with complications usually have low numbers of naive CD4 T cells but increased activated CD4 T cell counts (28–30), defective T cell functions (lower proliferative capacities, abnormalities in cytokine production) and reduced levels of regulatory T cells (31). Given their function as B helper cells, TFH represent a CD4 T cell subset of great interest in CVID pathogenesis and will now be discussed.

OVERVIEW OF TFH CELL FUNCTIONS

TFH are a CD4 T cell subset specialized in providing B cell help. They are essential for B cell differentiation into Ig-producing plasma cells and for generation of memory B cells. TFH are characterized by a unique set of molecules associated with their functions. The hallmark of TFH is CXCR5 expression, which allows their migration into GC follicles of secondary lymphoid organs through the attractive effect of the CXCL13 chemokine (32–34). Moreover, they express the transcription factor B cell lymphoma 6 (BCL-6), the co-receptors CD40L, programmed cell death 1 (PD-1) and ICOS, and they produce IL-21 (34), all of which being involved in their functions.

Mouse models have led to a better understanding of TFH biology over the past decade and these discoveries have already been reviewed (34–37). Here, we will focus on human TFH and their subsets. In fact, recent studies have considerably increased our knowledge of the human counterpart. The discovery of human circulating TFH within the memory CD4 T cell compartment has enabled a better understanding of these cells, since access to blood samples is much easier than access

to secondary lymphoid organs such as spleen from cadaveric organ donors or tonsils from children (38). They are considered as memory cells and reflect the *bona fide* TFH present in GC counterparts, even if they lack BCL-6 and ICOS expression. Interestingly, a recent and elegant study from Vella et al. comparing TFH from LN, thoracic duct lymph and blood shows that these cells share TCR clonotype, phenotype and transcriptional signatures, thus reinforcing the idea that the examination of circulating cells reflects what happens in GC (39). Based on the expression of the chemokine receptors CXCR3 and CCR6, Morita et al. have identified three subsets of TFH harboring different functions and affiliated with the classical helper subsets Th1, Th2, and Th17 (38) (**Table 1**). TFH1 are $CXCR3^+CCR6^-$, express T-bet and produce IFNγ; TFH2 are $CXCR3^-CCR6^-$, express GATA3 and produce IL-21 and IL-4; and TFH17 are $CXCR3^-CCR6^+$, express RORγT and produce IL-21 and IL-17A. More importantly, these subsets are divided into two groups based on their B helper cell functions, in particular their capacity to induce naive B cells to produce Ig: TFH2 and TFH17 are considered efficient helper cells, while TFH1 are non-efficient helpers (38, 42, 43). Based on CCR7, PD-1 and ICOS expression, these subsets can be further divided into different functional subpopulations, leading to the proposition by Ueno's group to include all these markers for human blood phenotyping of TFH (44, 45). $ICOS^+PD\text{-}1^{high}CCR7^{low}$ TFH are activated and could be considered as effectors. For instance, following influenza vaccination, TFH1 (known as non-helpers) can be activated to express ICOS and high levels of PD-1, also correlating with antibody responses. This means that they are able to help memory B cells *in vitro,* showing then a limited B helper cell function (46). Similarly, $CXCR3^+$ TFH expressing high levels of PD-1 correlate with neutralizing antibody responses in HCV patients (47). In contrast, Martin-Gayo et al. reported that neutralizing antibodies in HIV controllers correlate with the presence of $CXCR3^+PD1^{low}$ TFH, but that these cells might be precursors of $PD\text{-}1^{high}$ cells (48). Another subset of TFH, the T follicular regulatory cells (TFR cells) comprising a population of natural regulatory T cells that express FoxP3, BCL-6, and CXCR5, has been identified in mice. This subset seems important for the regulation of the GC reaction by limiting the number of TFH and B cells in GC or terminating the GC response (49–51). The biology of human TFR cells is not well-known. In human tonsils, the number of $FoxP3^+$ TFR in GC is lower than it is in mice (35). Circulating $FoxP3^+$ Tfr have been described (40). Cañete et al. identified a population of IL-10 producing human TFH expressing CD25 but lacking FoxP3 in tonsils and capable of dampening IgE responses, thereby suggesting a possible role for these cells in atopic diseases (41). Altogether, despite several studies focused on TFH biology over the past decade, the functions of each human subset are not fully discovered yet.

Mouse TFH differentiation is a multi-step process involving several signals, with a priming by dendritic cells (DC), or eventually B cells (52), in the T cell zone of secondary lymphoid organs, followed by migration of the pre-TFH to the T-B border and maturation into *bona fide* GC TFH requiring B cells (53). Human TFH differentiation has yet to become fully understood. IL-12 (54, 55), TGFβ (56), Activin A (57), and OX40L (58, 59) are key regulators of this process. Dermal $CD14^+$ DCs have been found as the best skin DC subset to drive TFH differentiation (60). Others have identified $CD1a^+$ dermal DCs and Langerhans cells as able to polarize CD4 T cell into IL-21 producer cells (61, 62). Recently, Durand et al. have uncovered tonsil cDC2 as the best TFH polarization inducer among the DC subsets they tested, and have shown that the interaction with tonsil macrophages located in B cell follicles is necessary for optimal TFH function (63).

TABLE 1 | Main characteristics of circulating TFH subsets.

	TFH1	TFH2	TFH17	TFR
B helper function	±	+	+	
Surface marker	CXCR3	–	CCR6	$CD25^{high}$ $CD127^{low}$
Transcription factor	T-bet	Gata3	RORγT	$FoxP3^{\pm *}$
Cytokine profile	$IL21^{low}$ IFNγ	IL21; IL4; IL13	IL21; IL17; IL-22	IL-10

The main characteristics of the circulating $CD4^+CD45Ra^-CXCR5^+$ follicular helper T cell subsets are described.
**Both FoxP3+ and FoxP3 TFR have been reported (40, 41).*

TFH are involved in numerous biological processes of health and disease, as reviewed in Ueno et al. (35), Crotty (36), and Ma and Deenick (64). They are involved in protection against numerous pathogens through the induction of Ab responses and vaccine-induced immunity, as well as in autoimmune diseases or HIV infection. The role of TFH in human primary immunodeficiency has already been well documented and reviewed (64, 65). For instance, distinct monogenic mutations in *STAT3*, *CD40LG*, *BTK*, *IL10R,* or *NEMO* that lead to different types of primary immune deficiency are associated with decreased circulating TFH number (66).

TFH AND CVID

As mentioned earlier, CVID is defined by B cell defects leading to low levels of serum Ig and impaired Ab responses. Nevertheless, defects in other immune cells are also present. Given their role as B helper cells, it is of interest to analyze TFH subsets in CVID patients. One series of evidence for TFH involvement in CVID pathogenesis is given by genetic analysis. The most striking is the rare deficiency in inducible T-cell COStimulator (ICOS), a co-receptor expressed by T cells. In these patients, B cells are genetically normal but do not receive optimal help from T cells, which leads to impaired T-cell dependent B-cell activation, absence of memory B cells, and failure in class-switching leading to hypogammaglobulinemia (67–69). Warnatz et al. studied nine patients with ICOS deletion and showed that combining all clinical features of the patients outlines the full range of associated complications to CVID (69). Interestingly, Bossaller et al. showed that ICOS deficiency is associated with a defect of TFH in germinal centers (68), showing that ICOS is essential for TFH generation in humans as well as in mice (70). Similarly, patients with a mutated *NFKB2* gene showed decreased levels of circulating TFH (71, 72). By contrast, Romberg et al. showed that a single *TACI* mutation leads to increased

levels of circulating TFH in CVID patients which correlate with levels of anti-nuclear antibodies suggesting that TFH may favor autoreactive B cell activation (73). Interestingly, Ellyard et al. also observed increased TFH, particularly circulating TFH1, in *TACI* mutant patients and of PD-1^{hi} $CCR7^{lo}$ TFH cells in CTLA4 mutant patients (74).

Interestingly, our group (75) and others (76–78) observed an increase of circulating TFH (memory $CXCR5^+$ CD4 T cells) in CVID patients harboring non-infectious complications. Moreover, TFH expressing PD-1 were present at higher levels in CVID patients with complications (75–78). Patients classified as smB^- based on the EUROClass have <2% of switched memory B cells among circulating $CD19^+$ cells (12). Interestingly, smB^- patients have higher levels of circulating TFH (77) [which is even more pronounced in the smB^- $CD21^{low}$ subgroup (78)] than smB^+ patients. The switched memory B cell population (IgG^+) contains some autoreactive B cells in normal adults (79), and $CD21^{low}$ memory B cells are increased in several autoimmune contexts (18). One can then hypothesize that smB cells in CVID patients, despite their low levels, contribute to autoimmunity, so TFH could participate to autoimmune manifestations through their role as smB cell inducers. Nevertheless, patients with autoimmune complications present similar levels of TFH or TFH subtypes to patients harboring other types of comorbidities (75), meaning that further experiments are needed to determine the impact of TFH on autoreactive Ab generation in CVID patients.

As explained earlier, TFH can be divided into two subsets: the non-efficient helper TFH1 and the efficient helpers TFH2 and TFH17. Interestingly, we (75) and others (77, 78, 80) highlight a specific increase of the circulating TFH1 only in non-infectious CVID patients. Moreover, $CXCR3^+$ (75) or T-bet^+ (78) cells were amplified in secondary lymphoid organs of CVID patients, suggesting that the blood observations reflect the GC counterpart. In contrast, Th17-oriented TFH were decreased. An increase in $CD25^+CD127^-CXCR5^+PD\text{-}1^+$ cells was observed, but these cells do not present regulatory functions and still need to be further characterized (80). TFH1 are not efficient B helper cells, partly due to their poor production of IL-21 (38). The combination of IL-21 and CD40 stimulation is able to restore Ig production and to improve memory B cell survival in *in vitro* settings using cells from CVID patients (81, 82). Moreover, addition of IL-4 and IL-21 (cytokines produced by TFH2) improved IgG production in some patients (83). Thus, the imbalance between TFH subsets, stable over time (75), could lead to poor IgG production. As TFH1 are good IFNγ producers and are increased in patients, one may hypothesize involvement of this cytokine in CVID pathogenesis. Surprisingly, even though two groups observed enhanced IFNγ production by TFH in CVID patients (77, 78), Le Coz et al. did not, rather finding increased IL-21^+ cells and accordingly efficient helper B cell function in CVID TFH despite observing a TFH1/TFH2-17 imbalance (80). Moreover, studies on putative IFNγ function in CVID are also puzzling. In fact, Desjardin et al. reported that addition of IFNγ to cultured B cells from CVID patients did not modulate IgG production (83), while Unger et al. showed that exogenous IFNγ reduced IgG and IgA production in T/B co-cultures (78). Moreover, the impact of IFNγ on $CD21^{low}$ cell generation and/or on autoreactive B cell activation has not been directly addressed, therefore still awaiting determination. Altogether, these data highlight that more experiments are necessary to determine TFH1 functions and putative IFNγ implication in the diverse clinical manifestations of CVID.

A question one may ask is the origin of the skewed TFH populations in CVID patients. A recent study from Le Coz et al. highlighted that part of the naïve CD4 T cells from CVID patients with autoimmune cytopenias (AIC) are skewed toward a follicular commitment based on their expression of specific markers (CXCR5, PD-1, CCR7, CD38, ICOS, T-cell factor 1). In addition, some recently identified thymic emigrant cells (defined as $CD45RA^+CD31^+$) express CXCR5 and PD-1 in CVID patients with AIC (80). These data suggest that CD4 T cells present follicular aspects as early as thymic egress stage. Moreover, TFH can differentiate from naive $CD4^+$ T cells by interacting with different dendritic cell subsets or under the influence of several cytokines such as IL-12 (55), TGFβ (56) or Activin A (57). Notably, Martinez-Pomar et al. reported high amounts of IL-12 in the sera of CVID patients (84), which was not confirmed by Le Coz et al. (80). By contrast, they found an increase in plasma levels of Activin A, correlating with circulating TFH frequencies. They also observed increased ICOSL expression on monocytes and demonstrated that endotoxemia is involved in TFH differentiation in CVID patients with AIC (80). Altogether, despite recent studies, the mechanisms leading to the imbalance of TFH1 vs. TFH2/TFH17 in CVID patients still need to be fully decoded.

CONCLUSION

Evidence from the literature strongly suggests a role for TFH in pathogenesis of the more severe forms of CVID, but more experiments are necessary to determine the mechanisms involved. A better understanding of these mechanisms would be of great interest to apprehend the immune context in CVID patients harboring non-infectious complications.

AUTHOR CONTRIBUTIONS

CL and DD wrote and edited the manuscript. SL, PB, and J-FV contributed to writing and critically revised the paper. All authors read, corrected, and approved the final manuscript.

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Non-infectious Complications of Common Variable Immunodeficiency: Updated Clinical Spectrum, Sequelae, and Insights to Pathogenesis

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Non-infectious complications in common variable immunodeficiency (CVID) have emerged as a major clinical challenge. Detailed clinical spectrum, organ-specific pathologies and associated sequelae from 623 CVID patients followed in New York since 1974 were analyzed, and recent insights to pathogenesis were reviewed. Non-infectious manifestations were present in 68.1% of patients, and they do not tend to be present in isolation. They include autoimmunity (33.2%), chronic lung disease (30.3%), lymphoid hyperplasia/splenomegaly (20.9%), liver disease (12.7%), granulomas (9.3%), gastrointestinal disease (7.3%), lymphoma (6.7%), and other malignancies (6.4%). In the lungs, interstitial disease and bronchiectasis were the most common findings, with lymphoma at this site being a rare ($n = 6$), but serious, manifestation. Bronchiectasis was not a prerequisite for the development of interstitial disease. In the liver, granulomas and nodular regenerative hyperplasia were the most common. Gastrointestinal disease may affect any segment of the intestinal tract, with lymphoid infiltrations and villous blunting being the leading histologic findings. With progression of organ-specific diseases, a wide spectrum of associated sequelae was observed. Lymphoma was more common in females ($P = 0.036$)—all B cell types except in one subject. Solid organ transplantations (liver, $n = 5$; lung, $n = 4$; combined lung and heart, $n = 2$) and hematopoietic stem cell transplantations (for B cell lymphoma, $n = 1$) have rarely been performed in this cohort, with mixed outcomes. Recent identification of monogenic defects, in ∼10–30% of various CVID cohorts, has highlighted the molecular pathways that can affect both antibody production and broader immune regulation. In addition, cellular defects in both innate and adaptive immune systems are increasingly recognized in this syndrome.

Keywords: common variable immunodeficiency, autoimmunity, immune dysregulation, thrombocytopenia, hemolytic anemia, granulomatous disease, interstitial lung disease, enteropathy

INTRODUCTION

Common variable immunodeficiency (CVID) is considered a primary defect, characterized by reduced serum levels of immune globulin (Ig) G, IgA, and or IgM, with reduced or absent specific antibody production (1–4). The diagnosis excludes secondary causes of hypogammaglobulinemia and other well-defined primary immunodeficiencies, including combined immunodeficiencies. It was first recognized by Sanford et al. in 1954 (5). With an estimated prevalence of 1:50,000–1:25,000, it is the most common symptomatic primary immunodeficiency. CVID patients share a central failure in B cell differentiation into functional Ig-secreting plasma cells, and while classified among B-cell defects, an increasing number of cellular defects have been recognized in recent years.

The clinical spectrum of CVID is broad but consist of two main phenotypes: one group with predominantly recurrent infections and a second group with additional autoimmune/inflammatory manifestations. These non-infectious complications may be evident at presentation or may appear afterward, and they include progressive lung disease, autoimmunity, gastrointestinal inflammatory disease, granulomatous disease, liver disease, lymphoid hyperplasia and infiltrative disease, and the development of cancer, especially lymphoma (6–8). This phenotypic distinction has important clinical implication because the risk of death is estimated at 11 times higher for patients with non-infectious complications compared to those without (7, 9). In addition, while the introduction of Ig replacement therapy has greatly reduced the number of infections (10), it does not appear to prevent or ameliorate most inflammatory and autoimmune conditions (6, 7). Non-infectious complications in CVID are therefore emerging as a major challenge, requiring a better understanding of underlying pathogenesis and additional therapeutics.

Recent investigations of this phenotypic syndrome have led to the discovery of a number of monogenic defects, in ∼10–30% of CVID patients, providing potential insights into both pathogenesis and more direct therapies (11–13). Broad innate and adaptive immune dysregulation are also increasingly identified, especially in subjects with non-infectious complications. In the clinics, rituximab has been shown to be effective for the treatment of autoimmune cytopenia in CVID (14), while additional immune modulators and biologics are becoming more widely utilized, with benefits in some cases (15–18).

Large cohort and registry data have provided key insights into non-infectious complications of CVID (6–8, 19–24). Previously, we have found that not all such complications were equally deleterious in our cohort. Increased morbidity risk is closely associated with a number of organ-specific pathologies, such as lung impairment, liver, and gastrointestinal disease, as well as lymphoma (7). Additional cohort studies have shown that several conditions, such as autoimmunity, granulomatous infiltrations, lymphoproliferation, and enteropathy appears to be clinically interrelated, suggesting some level of shared pathogenesis (8, 19). Here, we provide an updated clinical spectrum of non-infectious complications from 623 patients with CVID followed at our center, with delineated organ-specific pathologies and associated sequelae. In addition, we highlight recent efforts in dissecting monogenic defects and dysregulated immune pathways in CVID.

NON-INFECTIOUS COMPLICATIONS: UPDATED CLINICAL SPECTRUM AND SEQUELAE

Demographics and Immunologic Parameters

We detail the clinical spectrum, organ-specific pathologies, and associated sequelae from a cohort of 623 patients (277 males, 346 females) confirmed as having CVID based on standard criteria (1) in a patient cohort seen at Mount Sinai Hospital (1986–2019) and/or before this at the Memorial Sloan-Kettering Cancer Center (1974–1986). This study was approved by the Mount Sinai Hospital Institutional Review Board. The median age of symptom onset (major infection or characteristic non-infectious manifestation) was 25 years for males and 28 years for female. Consistent with prior reports, males were diagnosed with CVID earlier (median age of 30 years for males vs. 33.7 years for female). Overall, 18% of patients were diagnosed under the age of 21.

Median serum immunoglobulin levels at diagnosis were IgG, 237 mg/dL; IgA, 7 mg/dL; and IgM, 20 mg/dL. Serum IgG was <100 mg/dL in 24.7%; IgA was <7 mg/dL in 49.2%, IgM was <25 mg/dL in 55.9%. For subjects examined in this way, peripheral B cells were <1% of total lymphocytes in 7.6%; isotype switched memory B cells were <0.55% of total B cells in 35% (**Table 1**).

Overview of Non-infectious Complications

In our CVID cohort, 68.1% of the patient had one or more non-infectious complications (**Table 2**). Consistent with prior reports (8, 20, 21), the most common manifestation was autoimmunity

TABLE 1 | Immunologic parameters.

	Normal range*	Median (range)
IgG (mg/dL)	700–1,600	237 (UD−687)
IgA (mg/dL)	70–400	7 (UD−255)
IgM (mg/dL)	40–230	20 (UD−945)
T-cell populations		
CD3+, % ($n = 359$)	55–89	75.5 (16–98)
CD3+, cells/mm^3	750–2,500	1,080 (160–5,383)
CD3+CD4+, cells/mm^3 ($n = 254$)	480–1,700	633 (76–2,828)
CD3+CD8+, cells/mm^3 ($n = 200$)	180–1,000	381 (26–3,247)
B-cell populations		
CD19+, % ($n = 410$)	5–15	9 (0–58)
CD19+, cells/mm^3	75–375	146 (0–840)
Isotype-switched memory B cells (CD19+CD27+IgD–), % ($n = 223$)	6.5–29.2	1 (0–29)

**Normal range listed is for adults. UD, undetectable. Percentage of CD3+ T cells and CD19+ B cells are expressed as % of total lymphocytes; Percentage of isotype-switched memory B cells (CD19+CD27+IgD–) are expressed in percentage of total CD19+ B cell population.*

(33.2%, $n = 207$), with hematologic autoimmunity being the most prevalent (21.7%, $n = 135$). The most common organ-specific manifestation was functional or structural chronic lung diseases (30.3%, $n = 189$), followed by gastrointestinal diseases (17.3%, $n = 108$), and liver diseases (12.7%, $n = 79$). Lymphoid hyperplasia and/or splenomegaly was also common, with a prevalence of 20.9% ($n = 130$) in this cohort. Lymphoma was confirmed in 42 patients (6.7%), while other solid organ cancers was found in 40 patients (6.4%). Granulomatous disease was confirmed by biopsy in 58 patients (9.3%). Non-infectious complications did not tend to occur in isolation. Amongst those with such conditions, the majority (60.8%) experienced two or more non-infectious manifestations in their lifetime.

The prevalence of autoimmunity, chronic lung disease, gastrointestinal disease, and granulomatous disease was comparable to previously published cohort data (**Table 2**). Based on existing cohorts, the prevalence of autoimmunity ranged from 20.3 to 33.2%, chronic lung disease ranged from 30.3 to 46.4%, gastrointestinal disease ranged from 9 to 22.4%, and granulomatous disease ranged from 8 to 20%. The overall incidence of lymphoma was higher in our cohort (6.7%) compared to most prior reports (8, 20, 23, 25), though the reasons behind this are unclear.

Chronic Lung Disease and Associated Complications

Lung failure has been a leading cause of death amongst CVID patients. The presence of functional or structural lung impairment is associated with increased mortality (hazard ratio 2.06) (7). However, not all forms of lung disease appear to be equally deleterious, as increased mortality risk has not been observed in those with radiographic evidence of bronchiectasis alone (7). It is thought that the pathogenesis of bronchiectasis may be fundamentally different than other forms of chronic lung disease in CVID, with bronchiectasis being more closely related to tissue damage from recurrent pulmonary infections rather than broader immune dysregulation (26).

Chronic lung disease was the most common organ-specific complication in our cohort ($n = 189$, 30.3%). To provide better delineation of distinct CVID-associated lung diseases, we reviewed existing radiography and pathology reports in the cohort. Specific radiographic and/or biopsy-based diagnosis was available in 124 patients (**Figure 1A**). Amongst this group, the prevalence of interstitial lung disease (ILD) was 62.9% [$n = 78$; ILD was defined as computed tomography (CT)

TABLE 2 | Non-infectious complications.

	No.	% of cohort ($n = 623$)	Chapel et al. (8) ($n = 334$)	Quinti et al. (20) ($n = 224$)	Wehr et al. (21) ($n = 303$)	Farmer et al. (24) ($n = 205$)
Infection only	199	31.9	26%	NR	NR	NR
Non-infectious complication	424	68.1	74%	NR	NR	NR
Autoimmunity	207	33.2	NR	25.9%	20.3%	NR
Chronic lung disease	189	30.3	NR	46.4%	NR	NR
Lymphoid hyperplasia/ splenomegaly	130	20.9	30%	26.4%**	40.5%**	25.9%**
Gastrointestinal disease	108	17.3	9%	22.4%***	NR	21.5%
Liver disease	79	12.7	9%*	NR	NR	9.3%
Granulomas	58	9.3	8%	NR	11.6%	20%
Lymphoma	42	6.7	3%	1.8%	NR	5%
Other malignancies	40	6.4	3%	4.5%	NR	22%****

*Categorized as hepatomegaly in the original study. **categorized as splenomegaly in the original studies. ***categorized as chronic diarrhea in the original study. ****categorized as solid organ malignancy in the original study. NR, Not reported.*

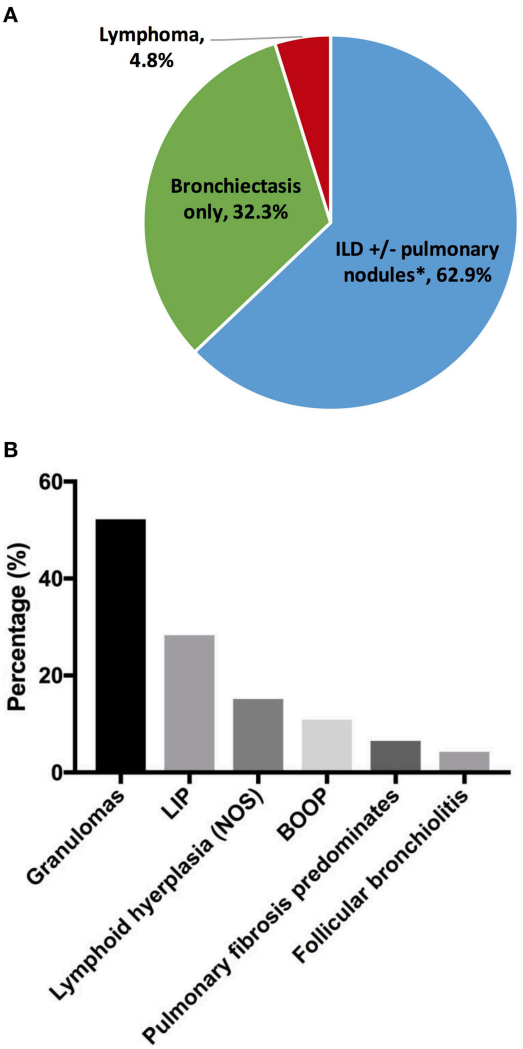

FIGURE 1 | Chronic lung disease. **(A)** Lung disease types by radiographs and/or pathology reports ($n = 124$). **(B)** Interstitial lung disease pathologies ($n = 46$). *Thirteen out of 65 subjects with ILD had concurrent bronchiectasis. ILD, interstitial lung disease; LIP, lymphoid interstitial pneumonia; BOOP, bronchiolitis obliterans organizing pneumonia.

evidence of ground glass opacities with or without more than 4 pulmonary nodules or mediastinal lymphadenopathy]. Radiographic evidence of co-existing ILD and bronchiectasis was observed in 10.5% ($n = 13$) of patients with lung disease, but the majority of patients with ILD ($n = 65$) did not have concurrent CT findings of bronchiectasis, indicating that the development of ILD was independent from the presence of bronchiectasis. The prevalence of isolated bronchiectasis, based on CT findings, was observed in 32.3% ($n = 40$). Lymphoma was diagnosed by lung biopsy in 6 subjects (4.8%), highlighting the necessity of tissue diagnosis in select cases to differentiate pulmonary nodules from malignancy.

Tissue histology may be useful to guide the selection of therapeutics for the distinct forms of interstitial disease (27). Biopsy reports were available in 46 subjects with ILD (**Figure 1B**). Amongst the subjects in this group, the most common pathology features were lung granulomas (52.2%, $n = 24$). Some forms of lymphoid infiltration were found in 43.5% ($n = 20$) of the patients (lymphoid interstitial pneumonia, 28.3%; lymphoid hyperplasia, not otherwise specified, 15.2%). Extensive lymphoid infiltrations and granulomas may be observed concurrently in some patients (and this was specified in 6 subjects, 13%). Features of bronchiolitis obliterans organizing pneumonia were found in 10.9%, and follicular bronchiolitis was found in 4.3%. In 3 subjects (6.5%), extensive pulmonary fibrosis was the predominant finding at the time of biopsy.

Chronic lung disease may lead to significant morbidity, including progressive structural and/or functional decline, as well as chronic oxygen supplementation requirement. Further complications may also develop from either lymphocytic interstitial lung disease, granulomatous lung disease, or bronchiectasis. Pulmonary hypertension was observed in 5.3% ($n = 10$) of the subjects with lung disease. This complication may arise from diverse lung pathologies (interstitial lung disease, $n = 2$; granulomatous lung disease, $n = 2$; bronchiectasis $n = 1$; lung pathology not-specified, $n = 5$). Six of these subjects subsequently developed cor pulmonale.

Six patients underwent lung transplantation. An additional patient underwent combined lung and liver transplant, but follow-up data were unavailable. Clinical information, including primary lung disease, comorbidity, and outcome, is summarized in **Table 3**. Patient 1 experienced hyperacute rejection and died within 3 days of transplantation. Patient 2 experienced acute rejection and died of associated complications after 8 months. Patient 3 had known CVID-associated haplotype HLA A1-B8-DR3 and two additional family members with a CVID phenotype. She presented with low IgG, low peripheral B cells and interstitial lung disease. After lung transplantation, she experienced acute rejection and died after 1 year. Patient 4 and 5 experienced chronic rejections and died 5 and 6 years following transplantation, respectively. Patient 6 had undergone lung transplant 4 months prior to the time of report.

Autoimmunity

Autoimmunity was observed in 33.2% ($n = 207$) of the overall cohort (**Table 4**). There was no gender difference in the prevalence of autoimmunity. As reported previously, the clinical spectrum was wide and includes both hematologic (21.7%, $n = 135$) and organ-specific autoimmunity (**Table 4**). Immune thrombocytopenic purpura (ITP) was the most common (16.2%, $n = 101$), followed by autoimmune hemolytic anemia (AIHA,

TABLE 4 | Autoimmune manifestations.

	No.	% of cohort ($n = 623$)
Hematologic autoimmunity	135	21.7
ITP	101	16.2
AIHA	48	7.7
Evans syndrome	29	4.7
Rheumatoid arthritis*	17	2.7
Anti-IgA antibody	8	1.3
Uveitis	6	1.0
Alopecia	5	0.8
Autoimmune thyroid disease	5	0.8
Others**	<5	<0.8

**additional 12 patients had non-specified arthritis. **Others (n < 5 each): pernicious anemia, anticardiolipin antibody, antiphospholipid syndrome, type 1 diabetes mellitus, uveitis, multiple sclerosis, systemic lupus erythematosus, lichen planus, vasculitis, vitiligo, psoriasis, myasthenia gravis, autoimmune pancreatitis, severe aphthous ulcer, autoimmune pancreatitis.*

TABLE 3 | Lung transplant outcomes.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age, sex	34, F	69, F	38, F	27, M	25, M	63, M
Lung pathology	Pulmonary fibrosis predominates	ILD (granuloma and lymphoid infiltrate), bronchiectasis	ILD	Chronic obstructive disease	Pulmonary fibrosis predominates	ILD
CVID-associated comorbidities	Liver disease	None	None	Enteropathy	Lymphoid hyperplasia	NRH, AIHA, ITP, lymphoid hyperplasia
Transplant procedure	Lung	Lung	Lung	Lung and heart	Lung and heart	Lung
Outcome	Died of hyperacute rejection within days	Died of acute rejection after 8 months	Died of acute rejection after 1 year	Died of chronic rejection after 5 years	Died of chronic rejection after 6 years	Alive 4 months post-transplant

ILD, interstitial lung disease; NRH, nodular regenerative hyperplasia; AIHA, autoimmune hemolytic anemia; ITP, Immune thrombocytopenic purpura.

7.7%, $n = 48$), consistent with prior cohort data (7, 8, 20). Eight patients were found to have anti-IgA antibodies. Other associated autoimmune conditions include rheumatoid arthritis (2.7%, $n = 17$) and uveitis (1%, $n = 6$). Rarer autoimmune complications (<1%) include alopecia, autoimmune thyroid disease, systemic lupus erythematosus, vasculitis, antiphospholipid syndrome, anticardiolipin antibody, psoriasis, multiple sclerosis, lichen planus, vitiligo, type 1 diabetes mellitus, and pernicious anemia. Myasthenia gravis, autoimmune pancreatitis, and severe oral aphthous ulcers ($n = 1$ each) were newly observed autoimmune conditions in CVID since our last report (7).

Gastrointestinal Inflammatory Disease and Malabsorption

Gastrointestinal disease and malabsorption in CVID are associated with increased mortality (HR = 2.78 and 2.06, respectively) (7). Varying degrees of enteropathy, which can mimic inflammatory bowel disease clinically, are commonly seen, and may affect any parts of the gastrointestinal tract. In our cohort, the prevalence of gastrointestinal disease was 17.3% ($n = 108$) overall. The presentation may be severe, with malnutrition (significant nutritional deficiency and/or total parenteral nutrition requirement) recorded in 35.2% ($n = 38$) of those with gastrointestinal manifestations. Comprehensive biopsies from upper and lower endoscopies were available in 34 subjects (**Table 5**). Amongst this group, disease involvement in the small intestines and large intestines was seen in 79.4% ($n = 27$) and 50% ($n = 17$), respectively. Absence or near absence of plasma cells was a common feature, specified in 47.1% ($n = 16$). In the small intestine, typical histologic findings included intraepithelial lymphocytosis (64.7%, $n = 22$), villous atrophy/blunting (32.4%, $n = 11$), nodular lymphoid hyperplasia (8.8%, $n = 3$), and non-specific inflammation (8.8%, $n = 3$). Granulomas were noted in one subject. CD8+ T cell infiltrates were specified in 3 subjects and mixed cellular infiltrates (neutrophils, eosinophils, histiocytes) were specified in 3 subjects. In the large intestine, non-specific inflammation (29.4%, $n = 10$), nodular lymphoid hyperplasia (11.8%, $n = 4$), and granulomas (8.8%, $n = 3$) were most commonly reported. Crypt abscesses were seen in one subject. While some histological findings (i.e., villous blunting) may resemble celiac disease, gene expressions analysis by microarray had previously indicated that they were likely distinct disease entities (28). In our cohort, 17 subjects with gastrointestinal disease had received celiac genetics testing, and only 3 subjects were found to carry celiac-associated HLA-DQ haplotype.

Gastric disease was noted in 58.8% ($n = 20$) of available reports. Gastritis and gastropathy (not otherwise specified) were seen in 7 subjects each. Lymphoid aggregates were observed in 3 subjects, and granulomas were observed in 1 subject. Some degree of metaplasia was noted in 2 subjects. From esophageal biopsies, two subjects were found to have eosinophilic esophagitis, while one patient had non-specific inflammation.

TABLE 5 | Gastrointestinal disease pathologies by location.

	No.	% of group
Comprehensive biopsies available	34	100
Small intestine disease	27	79.4
Intraepithelial lymphocytosis	22	64.7
Villous atrophy/blunting	11	32.4
Nodular lymphoid hyperplasia	3	8.8
Non-specific inflammation	3	8.8
Granulomas	1	2.9
Large intestine disease	17	50.0
Non-specific inflammation	10	29.4
Nodular lymphoid hyperplasia	4	11.8
Granulomas	3	8.8
Gastric disease	20	58.8
Gastritis, NOS*	7	20.6
Gastropathy, NOS	7	20.6
Lymphoid aggregates	3	8.8
Metaplasia	2	5.9
Granulomas	1	2.9
Esophageal disease	3	8.8
Eosinophilic esophagitis	2	5.9
Esophagitis, NOS	1	2.9

NOS, not otherwise specified.

Liver Disease and Sequelae

In our previous report, we found that the presence of liver disease was also associated with increased risk of mortality (HR = 2.48) (7). Seventy-nine subjects (12.7%) were found to have liver disease in our cohort. Biopsy report was available in 40 subjects (**Figure 2A**). Amongst this group, granulomas (32.5%, $n = 13$) and nodular regenerative hyperplasia (NRH, 30%, $n = 12$) were the most common pathological features. Two patients were noted to have both granuloma and features of NRH. Nine subjects (22.5%) were noted to have general inflammation of the liver, with predominantly lymphoid infiltrates specified in 5 subjects. Primary biliary cholangitis was noted in 10% ($n = 4$), while primary sclerosing cholangitis was noted in 1 subject. Cirrhotic features predominated in 3 subjects at the time of biopsy.

Liver disease-associated sequelae were observed in 32.9% ($n = 26$) of the patients with known chronic liver disease (**Figure 2B**), and these may be severe with disease progression. These sequelae were equally likely to develop in either NRH or granulomatous liver disease in our cohort (two tailed $P = 1.0$, Fisher's exact test). Evidence of portal hypertension was seen in 32.9% ($n = 26$) of these subjects. Esophageal varices were found on endoscopy in 17.7% ($n = 14$), with variceal bleed recorded in 3 patients. Ascites was observed in 15.2% ($n = 12$), with spontaneous bacterial peritonitis noted in 3 subjects. Hepatopulmonary syndrome was recorded in 6.3% ($n = 5$), and all of the patients required chronic oxygen supplementation. Overt hepatic encephalopathy was noted in 2 subjects.

Five patients underwent liver transplant due to end stage liver failure. Clinical information, including primary liver disease, comorbidity, and outcome, was summarized in **Table 6**. Two patients (patient 1 and 2) suffered from acute rejection and died in the setting of organ failure and severe infections within 1 year. One patient (patient 3) died 2 years after transplant from

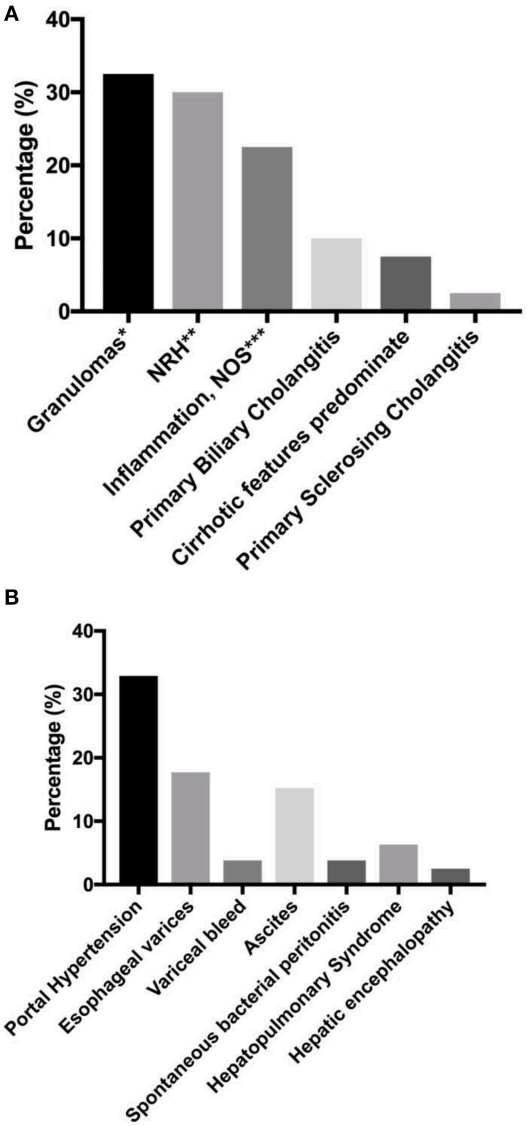

FIGURE 2 | Liver disease and associated sequelae. **(A)** liver disease pathologies ($n = 40$). **(B)** Liver disease-associated sequelae, reported as percentage of total number of subjects with known liver disease ($n = 79$). *Two patients with concurrent NRH. One patient with concurrent obliterative portal venopathy specified. **One patient with concurrent obliterative portal venopathy specified. *** lymphoid infiltrates predominate in 5 patients. NRH, nodular regenerative hyperplasia; NOS, not otherwise specified.

complications associated with infections. Two patients are alive 3 years (patient 4) and 6 years (patient 5) after transplant. Patient 5 had recurrence of granulomatous disease in the transplanted liver, and was re-listed for a second liver transplant for stage 4 cirrhosis at the time of report.

Lymphoma and Other Neoplastic Disease

Lymphoma in CVID has been shown to be associated with reduced survival (HR = 2.44) (7). Forty-two patients (6.7%) had a lymphoid malignancy in this cohort. Lymphoma was significantly more common in females ($n = 30$, 8.7% of female subjects) than males ($n = 12$, 4.3% of male subject; two-tailed $P = 0.036$, Fisher's exact test), as noted previously. Detailed pathology reports were available in 39 subjects (**Table 7**). All lymphomas recorded were B cell in type, with the exception that one patient had ALK negative anaplastic large cell lymphoma (**Table 7**). Amongst this group, the vast majority of lymphomas were non-Hodgkin's lymphoma (89.7%, $n = 35$). Hodgkin's disease was noted in 3 subjects (7.7%), and all 3 patients subsequently developed secondary lymphoma of unknown B cell type. Various types of B cell lymphoma were noted in the cohort, including diffuse large B cell lymphoma ($n = 10$), T cell rich B cell lymphoma ($n = 3$), plasmacytoid lymphoma ($n = 1$), marginal zone lymphoma ($n = 5$), and extranodal marginal zone lymphoma of MALT ($n = 1$). A few biopsies were categorized under the Working Formulation classification at the time of original pathology report, and they included diffuse mixed small and large cell lymphoma ($n = 2$), diffuse small cleaved cell lymphoma ($n = 1$), diffuse poorly differentiated lymphoma ($n = 1$), and follicular mixed cell lymphoma ($n = 1$). Ten patients had non-Hodgkin's lymphoma of B-cell type that was not further classified. Solid organ malignancies were seen in 40 subjects (6.4% of overall cohort, **Table 8**). Two patients developed 2 distinct primary malignancies (one subject with colon and prostate cancer, and one subject with prostate and skin cancer). One patient underwent allogenic hematopoietic stem cell transplantation (HSCT) for B cell lymphoma (not otherwise specified), but outcome data were unavailable.

Granulomatous Disease and Lymphoid Proliferation

The morbidity and mortality impact of granulomatous disease in CVID may be dependent on its location. While the presence of granuloma in general was not previously associated with shorter survival, granulomas found in lungs and liver may lead to tissue destruction and organ-specific sequelae, as observed here and previously (7). These granulomatous changes may be mistaken as "sarcoidosis," leading to delayed recognition of CVID. In prior reports, the association of granuloma, autoimmunity, and splenomegaly had been noted (8, 20). Granulomatous disease diagnosed by tissue biopsy was seen in 58 subjects (9.3% of overall cohort), and they typically consisted of well-formed non-caseating granulomas. This prevalence is likely an under-estimation as many patients did not undergo tissue biopsy. Granulomas may occur in a wide variety of organs (**Table 9**). The most common sites of granulomas identified by biopsies included lung ($n = 25$), liver ($n = 13$), skin ($n = 10$), and lymph nodes ($n = 9$). Rare but notable locations included brain ($n = 2$), bone marrow ($n = 1$), parotid gland ($n = 3$), and the mesentery ($n = 1$, presented as a large mesentery mass).

Lymphoid hyperplasia and/or splenomegaly were common features, seen in 130 subjects (20.9% of overall cohort). Splenomegaly was recorded in 97 subjects, while lymphadenopathy was recorded in 56 subjects. Twenty-three

TABLE 6 | Liver transplant outcomes.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age, Sex	54, F	46, M	48, M	46, M	40, F
Liver pathology	Primary sclerosing cholangitis, cholangiocarcinoma	Hepatitis (non-A, non-B)*	Primary biliary cholangitis	Nodular regenerative hyperplasia	Granulomatous liver disease
CVID-associated conditions	ITP, AIHA	Chronic lung disease	Lung granulomas, splenomegaly	Bronchiectasis	AIHA, s/p splenectomy
Transplant procedure	Liver	Liver	Liver	Liver	Liver
Outcome	Died of acute rejection within 1 year	Died of acute rejection within 1 year	Died of infections after 2 years	Alive 3 years post-transplant	Alive 6 years post-transplant, recurrence of granulomas in transplanted liver

**Before availability of hepatitis C virus PCR. AIHA, autoimmune hemolytic anemia; ITP, Immune thrombocytopenic purpura.*

TABLE 7 | Lymphoma types.

	No.
Detailed pathology available	39
Non-Hodgkin's lymphoma	35
B-cell type, not otherwise specified*	10
Diffuse large B cell lymphoma	10
T cell rich B cell lymphoma*	3
Plasmacytoid lymphoma	1
Marginal zone lymphoma	5
Extranodal marginal zone lymphoma of MALT	1
Diffuse mixed small and large cell lymphoma	2
Diffuse small cleaved cell lymphoma	1
Diffuse poorly differentiated lymphoma	1
Follicular mixed cell lymphoma	1
Hodgkin's disease**	3
Anaplastic large cell lymphoma (ALK -)	1

**One patient with +EBV in each category. **All 3 patients subsequently developed secondary lymphoma of unknown B cell type.*

TABLE 8 | Other cancers.

	No.
Other cancers	40
Breast cancer	9
Colon cancer	3
Lung cancer	3
Ovarian cancer	3
Gastric cancer	3
Melanoma	3
Cholangiocarcinoma	2
Oral cancer	2
Skin cancer	2
Thyroid cancer	2
Colon, prostate cancer	1
Esophageal cancer	1
Hepatic carcinoid tumor	1
Meningioma	1
Pituitary adenoma	1
Prostate, skin cancer	1
Testicular cancer	1
Vaginal cancer	1

subjects had concurrent splenomegaly and lymphadenopathy. Fifty-four patients (8.7% of overall cohort) underwent a splenectomy due to either uncontrolled cytopenias (ITP or AIHA) or hypersplenism. Amongst this group, 3 patients (5.6%) had documented sepsis due to an infection after splenectomy. Two additional patients had been hospitalized post-splenectomy for meeting clinical criteria for sepsis, though no positive microbiology was recorded. One of these 5 patients were not on immunoglobulin replacement at the time of sepsis. Rare additional complications had been noted in patients who had undergone splenectomy. As reported previously, 2 subjects developed fistulas to other organs or the exterior skin, and 2 subjects developed unexplained portal hypertension and secondary liver failure (7). Pulmonary arterial hypertension had previously been linked to post-splenectomy status in other diseases (29). Three out of 10 subjects with pulmonary arterial hypertension in our cohort had a history of splenectomy, though with concurrent chronic lung disease in all 3 patients, it was unclear whether splenectomy was a major contributing factor.

INSIGHTS TO THE PATHOGENESIS OF NON-INFECTIOUS COMPLICATIONS IN CVID

Monogenic Defects in Immune Regulation and B Cell Function

In the past 2 decades, genetic studies of the CVID phenotype have led to the successful identification of a number of monogenic defects (11–13). In our New York cohort, a monogenic etiology has been assigned to as many as ∼30% of the subjects (12). Two broad categories of genetic defects have emerged from these studies. The first includes mutations in genes that are involved in various stages of B cell activation, survival, and maturation to the plasma cell stage. In the second category,

TABLE 9 | Granulomatous disease by locations.

	No.
Granulomas (total)	58
Lung	25
Liver	13
Skin	10
Lymph node	9
Eye	3
Brain	2
Gastrointestinal tract	2
Oral	2
Parotid gland	2
Soft tissue	2
Spleen	2
Bone marrow	1
Kidney	1
Mesentery mass	1

defects in genes that control general immune regulation are also increasingly recognized in subjects with the CVID phenotype. In the later, additional features of autoimmunity and inflammation are commonly seen. Together, these monogenic defects have provided key insights to the molecular pathways that can lead to concurrent antibody deficiency and broader immune dysregulation in select patients, along with targeted therapies in some cases. The main genes linked to this syndrome and the associated clinical phenotype are discussed here.

Amongst immune regulatory genes, pathogenic variants of nuclear factor kappa B subunit 1 (NF-kB1) are the most common defects in the US and European CVID cohorts (12, 30). The NF-kB family of transcriptional factors includes 5 related proteins, c-Rel, p65 (RelA), RelB, p50 (NF-kB1), and p52 (NF-kB2), which forms various homo- and heterodimers to regulate the expression of a wide range of target genes (31). The NF-kB signaling pathway is involved in diverse processes, notably including B cell differentiation and function, as well as immune response to microbial and inflammatory stimuli. NF-kB1 defects identified in CVID cohorts exhibit autosomal-dominant inheritance with variable penetrance (32–35). These patients present with antibody deficiency and a B cell phenotype that is characteristic of CVID. Clinically, non-infectious manifestations are common, reflecting the broader impact of dysfunctional NF-kB signaling. These include autoimmunity, lymphoid hyperplasia, lung disease, enteropathy, liver disease, granulomas, and malignancy (30, 32–35). Autosomal dominant, heterozygous NF-kB2 defects have also been identified in CVID cohorts (36). These patients present with early onset hypogammaglobulinemia, infections, along with autoimmune features, and notably, adrenal insufficiency. Other non-sense gain-of-function NF-kB2 mutations have been identified in 2 families, characterized by hypogammaglobulinemia, lymphopenia, and T-cell defects (37).

Lipopolysaccharide (LPS)-responsive beige-like anchor protein (LRBA) and cytotoxic T lymphocyte antigen 4 (CTLA4) are closely associated proteins primarily known for the regulation of T cell response, but mutations in both genes have been identified in CVID cohorts (12). LRBA is localized in the vesicles and endoplastic reticulum, with domains homologous to vesicle trafficking proteins. Its function is closely linked to CTLA4 as it prevents lysosomal degradation of CTLA4 (38). As such, LRBA deficient patients have low CTLA4. CTLA4 is an inhibitory checkpoint protein on activated T cells and regulatory T (Treg) cells (39). It exerts immune-modulatory effects by competing with the T cell co-stimulatory molecule CD28 for the ligands CD80 and CD86 on antigen presenting cells (APC), or by removing these ligands by trans-endocytosis, resulting in reduced APC-mediated T cell activation. CTLA4 is essential for the function of Treg cells, which are responsible for maintaining self-tolerance and immune homeostasis. Homozygous recessive LRBA mutations have mostly been identified in childhood onset disease, but compound heterozygous mutations have been identified in adult CVID cohorts (40–42). In LRBA deficient patients, there are concurrent B cell defects (disturbed development, defective activation, poor plasmablast formation) manifesting as hypogammaglobulinemia, and T cell dysregulation (decreased Treg cell markers, expansion of T follicular helper cells, contraction of T follicular regulatory cells) that may contribute to inflammatory complications (38, 42). Clinically, these patients are affected by recurrent infections, autoimmunity, lymphoid hyperplasia, as well as severe inflammatory bowel disease in some (40–42). Heterozygous CTLA4 mutations, on the other hand, can lead to haploinsufficiency, impaired protein dimerization, or impaired ligand binding, causing an autosomal dominant syndrome with variable penetrance (43–46). Clinically, this highly variable syndrome is marked by antibody deficiency (84%), lymphoproliferation (73%), autoimmune cytopenia (62%), as well as lung (68%) and gastrointestinal (59%) diseases, amongst additional non-infectious complications, in a large cohort study ($n = 133$) (46). The elucidation of this molecular pathway and the availability of CTLA4 fusion proteins (abatacept, belatacept) have allowed for the potential for targeted treatment approach. In select cases, the loss of CTLA4 in LRBA deficiency has been successfully treated with abatacept (38). Similarly, some subjects with CTLA4 haploinsufficiency have had positive clinical response to abatacept or belatacept (11/14 subjects in one series) (46).

Phosphoinositide 3-kinase (PI3K) defect is another example of immune regulatory proteins that can lead to prominent antibody deficiency in the setting of broader dysregulation. PI3K contains multiple subunits, including the p110δ (PIK3CD) catalytic subunit and p85α (PIK3R1) regulatory subunit. PI3K is expressed predominantly in hematopoietic cells and is involved in signaling downstream of T and B cell receptors, toll-like receptors (TLRs), co-stimulatory receptors and cytokine receptors (47). It is therefore closely linked to the proliferation, survival, and activation of these cells. Dominant mutations in PI3KCD is now known as activated PI3Kδ syndrome (APDS), and some subjects may present with a CVID-like or hyper-IgM phenotype, with recurrent sinopulmonary infections, reduced IgG in 43%, reduced IgA in 50%, and increased IgM in 79%

of subjects (48–51). This syndrome has incomplete penetrance and variable expressivity. Clinically, it is also characterized by a high prevalence of non-infectious manifestations, including lymphoproliferation (75%), autoinflammatory disease (34%), and lymphoma (13%) (51). However, unlike most CVID subjects, herpesvirus infection is relatively common (49%) in these patients, and their immunophenotype is notable for reduced naïve T cells, increased highly differentiated effector/effector memory T cells, and expanded transitional B cells (51). Dominant heterozygous mutations in the p85α (PIK3R1) regulatory subunit of PI3K have also been reported in 4 subjects from 3 families (52). These lead to a similar syndrome of hyperactive PI3K signaling, with reduced IgG and IgA, variable IgM, and recurrent sinopulmonary infections, along with lymphoproliferation, autoimmunity, and terminally differentiated effector T cells.

Other notable monogenic defects that can result in concurrent antibody deficiency and non-infectious complications include: signal transducer and activator of transcription 3 (STAT3) gain-of-function (GOF) germline mutations, inducible T-cell costimulatory (ICOS) deficiency, IKAROS deficiency, and an interferon regulatory factor-2 binding protein 2 (IRF2BP2) mutation. STAT3 is a transcription factor, regulating multiple processes that includes cellular proliferation, differentiation, as well as autoimmunity. Patients with STAT3 GOF mutations have prominent early-onset multiorgan autoimmune and lymphoproliferative features, though more than half of the subjects (18 of 28) also have hypogammaglobulinemia in a recent systematic review (53). Common non-infectious manifestations include hematologic autoimmunities, enteropathy, interstitial lung diseases, and endocrinopathies (53–55). ICOS is a surface receptor of T cells. It is in the same family of proteins as CD28 and CTLA-4, and it is required for both T cell-APC interaction and T cell-B cell interaction in the germinal center. Homozygous and compound heterozygous mutations in ICOS are overall rare, but can lead to non-infectious complications that include enteropathy, autoimmunity, and hepatomegaly (56, 57). IKAROS is a hematopoietic zinc-finger transcription factor that is considered as master regulators of lymphocyte differentiation. In addition to hypogammaglobulinemia that can be profound, 2 subjects in the original cohort had B-cell acute lymphoblastic leukemia and 1 subject had thrombocytopenic purpura (58). IRF2BP2 is a transcriptional co-repressor that can affect cytokine production and it may also be involved in the development or survival of memory B cells. In the original report, variable degree of Ig deficiency, enteropathy, psoriasis, and type 1 diabetes were observed in a family with an IRF2BP2 mutation (59). In select cases, hypomorphic mutations in recombinase activating genes (RAG1 and RAG2) may also lead to prominent autoimmunity/hyperinflammation and antibody defects; however, these patients often have atypical features, such as opportunistic infections or severe systemic viral infections, suggesting a diagnosis other than CVID (60–62).

Deficiencies in B-cell costimulatory molecules are relatively rare, but can lead to a CVID phenotype. These defects are not known for broad inflammatory complications, but select non-infectious complications have been noted in a few cases. CD19 is a transmembrane protein expressed throughout B cell development until the plasma cells stage. It forms a complex with CD21 and CD81 on the surface of mature B cells and together, they are involved in signal transduction through the B cell receptor. Autosomal recessive mutations in these genes, and also in CD20, lead to defective activation of B cells and hypogammaglobulinemia. Amongst these disorders, nephropathy and thrombocytopenia associated with anti-platelet antibodies have been reported in a child with a homozygous CD81 gene defect (63), while chronic diarrhea and splenomegaly was reported in an adult with CD21 deficiency (64). Other B cell surface receptors defects, such as mutations in the B-cell activating factor of the tumor necrosis family (BAFF) receptor (BAFF-R) (65, 66), which is involved maturation of splenic B cells, and autosomal recessive mutations in CD27 (67), a receptor associated with memory B cells, may also lead to a CVID phenotype. However, autoimmunity and inflammatory complications have not been reported in these patients.

Lastly, in some cases, mutations in B-cell-specific genes have been heavily associated with autoimmunity and inflammation in CVID, though the molecular pathways leading to such a phenotype are not as obvious. Transmembrane activator calcium-modulating cyclophilin ligand interactor (TACI) is a product of the gene TNFRSF13B, and it is a receptor found on B cells. It binds to B-cell activating factor (BAFF) as well as a proliferation-inducing ligand (APRIL), and supports class-switch recombination, plasma cell differentiation, and antibody secretion during later stages of B-cell development. TACI mutations, usually in the heterozygous state, are enriched in the CVID population (8–10%) (68, 69), and they are significantly associated with autoimmunity and lymphoid hyperplasia, implying an additional role of TACI in the establishment of tolerance (70–72). However, heterozygous TACI mutations can also be seen in the healthy population, albeit at a much lower frequency, and phenotypically "normal" relatives of CVID subjects (73). Thus, potential functional impact of heterozygous TACI variants remains an area of active investigation.

The complexity of genetic defects in those with the CVID phenotype continues to be revealed. There are currently no definitive answers for when genetic studies should be recommended. While many investigations have focused on subjects with non-infectious complications, monogenic defects have also been identified in as many as ∼25% of those without in two large Western cohorts ($n = 395$ combined; in press). Disease severity may not necessarily correlate with the presence of a monogenic defect. In our cohort, no pathologic gene variants have been identified in patients with severe organ damage requiring transplantation. While there are no clear criteria, subjects with difficult-to-control autoimmunity, lymphocytic infiltrations in lungs/other organs, granulomatous disease, or lymphoma may benefit most from genetic investigations because such information may aid treatment selection. The clinical implication of monogenic defects for HSCT in CVID is currently not known; overall, HSCT rarely has been performed in this population, with mixed outcomes to-date (74).

Immunologic Abnormalities Associated With Non-infectious Complications

In addition to the identification of monogenic defects in a subset of patients, broad adaptive and innate immune dysregulation are increasingly recognized in CVID, especially in those with non-infectious complications. In the B cell compartment, a number of phenotypic changes have been observed, and classifying patients based on this framework has found clinical relevance (21, 75). Amongst CVID subjects, the prominent B cell abnormality is a reduction of isotype-switched memory B cells, possibly reflecting defective germinal center development in this syndrome. Using a cutoff of ≤0.55% of peripheral B cells, Sanchez-Ramon et al. have shown that a severe lack of isotype-switched memory B cells in CVID is an independent risk factor for granulomas, autoimmune disease and splenomegaly (75). In some CVID patients, there is also an expansion of $CD21^{low}$ B cells in the peripheral blood. These $CD21^{low}$ B cells have previously been reported in other autoimmune states, carrying unique characteristics that include an unmutated B-cell receptor as well as potentially being polyreactive (76). In CVID, the expansion of $CD21^{low}$ B cells (>10%) has been linked to splenomegaly (21). In a separate study, this phenotype is associated with autoimmune cytopenia as well (77). In another group of CVID subjects, an expansion of transition B cells has been identified. Notably, more than 50% of patients with this phenotype (defined as transitional B cells > 9%) has lymphadenopathy in one report (21). Lastly, in rarer cases, a near absence of B cells (<1% of total B cells) has been reported, suggesting severe defects of early B-cell differentiation in this group (21). An association between gender and distinct B cell profiles has also been described in CVID. In one study, female subjects are found to have higher levels of serum IgM and a greater number of isotype-switched memory B cells than male subjects (75), but whether this B cell phenotype differential is linked to the higher incidence of B-cell lymphoma in female CVID patients remains unknown.

In the T cell compartment, CVID is often characterized by broad and substantial T cell impairments, and in some cases, these defects are associated with overt clinical complications. As seen in our previous report (7), reduced T cell counts is observed in up to 29% of the cohort. In a minority (3.9%) of patients, severe CD4 T cell lymphopenia (<200 cu/mm) has been reported (7, 78). For these patients, an alternative diagnosis of late-onset combined immunodeficiency has been proposed as they can be prone to opportunistic infections (78). Functionally, impaired *in vitro* lymphocyte proliferation to both specific (antigen) and non-specific (mitogen) activators are observed in up to 50% of the CVID cohort (7). Impaired response to chemokines and abnormal lymphocyte trafficking have also been reported (79). Additional studies have shown that T cells in CVID often expresses activation markers and a marker associated with proliferation (Ki-67) (80); at the same time, they have a tendency to undergo apoptosis (81). However, the intrinsic and/or extrinsic drives behind these T cell impairments remain unclear. A reduction of regulatory T (Treg) cells, which are normally responsible for modulating immune response and maintain self-tolerance, has also been identified (77, 82). This phenotype is most profound in CVID subjects with reduced isotype-switched memory B cells and expanded $CD21^{low}$ B cells. Clinically, decreased Treg cells are associated with lymphoproliferation and autoimmune cytopenia, along with elevated inflammatory markers (77, 82). Another pervasive T cell abnormality in CVID is a clonal and constricted T cell repertoire, but they do not appear to be associated with specific clinical complications based on the current literature (83).

A wide range of cytokine defects have also been described in CVID; some of these have been more directly linked to specific cellular defects and/or clinical compilations, and will be highlighted here. First, a lack of IL-2 production has long been observed in CVID, and this was thought to contribute to poor T cell proliferation and function in this disorder. In early clinical trials of recombinant IL-2 treatment, some clinical and immunologic benefits of this approach were indeed observed (84–86). In contrast, IL-7, which may contribute to proliferation of autoreactive T cell clones, appears to be elevated in a subgroup of CVID subjects (87). In this group, elevated serum IL-7 correlates with increased CD8+T cells and a higher incidence of autoimmunity, but is not associated with T cell lymphopenia (87). More recently, elevated serum B cell-activating factor (BAFF) have also been reported in CVID and it may drive pulmonary B cell hyperplasia in patients with progressive interstitial lung disease (88). Other cytokine alterations reported in CVID include increased IL-6 (89–91), increased TNF-α (92), and variable IL-4 (90, 91).

Using whole blood mRNA transcriptional profiling, Park et al. have reported a marked up-regulation of interferon responsive genes in CVID subjects with inflammatory complications when compared to those without such complications and from control subjects (93). At the same time, modular analysis of the RNA transcript showed a greater reduction of both B and T cell networks in those with inflammatory manifestations (93). Together, these observations point to an impaired adaptive immunity that is coupled with chronic activation of innate interferon pathways in CVID subjects with inflammatory complications. In the follow-up study, IFN-γ, IL-17A, and IL-22+ cells with markers of innate lymphoid cells type 3 (ILC3; lineage negative, CD127+, CD161+, T-box transcription factor+, and retinoid acid-related orphan receptor γ+) were found to be expanded in the peripheral blood of CVID subjects with inflammatory conditions (mean 3.7% of peripheral blood mononuclear cells) (94). These cells had inflammatory potentials and they were also identified in the gastrointestinal and lung tissues of CVID patients with non-infectious organ disease, suggesting a role in mucosal inflammation (94). An additional study has similarly identified an expansion of ILC3, most pronounced in CVID subjects with autoimmune and/or lymphoproliferative complications, but also a relative loss of ILC2 (95).

While broad immunologic abnormalities and inflammatory complications in CVID are likely intrinsic to the underlying genetic and immune defects, a potential influence of environmental stimuli, namely commensal bacteria and their products, has been the subject of ongoing investigation in

recent years. Perreau et al. detected high levels of endotoxins in plasma of CVID subjects prior to starting Ig replacement therapy (96). This observation was associated with reduced proliferation capacity of bacteria-specific CD4 T cells and higher expression of programmed death 1 (PD-1) on CD4+ T cells, potentially suggesting a relatively "exhausted" phenotype. In this initial report, Ig replacement eliminated plasma endotoxin and reversed the CD4+T cells defects ascribed to the translocation of bacterial endotoxin (96). Thus, an alternative hypothesis for some CVID subjects, at least prior to Ig treatment, might be that chronic translocation of bacterial products could contribute to some levels of T cell impairment. Of note, the phenomenon of endotoxinemia in CVID has not been consistently detected in other reports, possibly due to the effects of Ig replacements, and it has not yet been directly linked to cellular activation (97, 98). At the same time, clinical data do not show that Ig therapy significantly alters the clinical course of most non-infectious complications (7). However, as we have come to better appreciate the bidirectional influence of host immunity and commensals on one another, and with increasing examples of their possible impacts in other autoimmune states (99, 100), the potential disturbance of host-commensal homeostasis and the associated immune consequences in CVID may warrant further evaluation.

CONCLUSION

While CVID is classified among the B-cell defects, additional cellular defects and immune dysregulation have been recognized in this syndrome over time. This is reflected clinically in the broad spectrum of non-infectious manifestations seen in a significant proportion of patients, which can lead to further sequelae with disease progression and increased mortality compared to those without such complications. The introduction of immunoglobulin replacement therapy has reduced the incidence of severe respiratory tract infections and associated mortality seen in the early years. However, with a lack of effective treatment in many cases, chronic non-infectious inflammatory and autoimmune conditions have emerged as challenging clinical problems in CVID. Recent genetic studies of this phenotype have led to the identification of monogenic defects in both B-cell centric genes and broader immune regulatory genes, providing insights to pathogenesis and potentially more targeted treatments in select patients. Moving forward, further genetic and immunologic understanding of this complex and heterogeneous syndrome is needed for the development of new therapeutic approaches.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Icahn School of Medicine at Mount Sinai Institutional Review Board. Written informed consent to participate in this study was provided by the participants or participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HH and CC-R conceived the study, collected data, and drafted manuscript. CC-R provided critical revisions of the manuscript and final approval of the version to be published.

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REVIEW
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Heterogeneity of Liver Disease in Common Variable Immunodeficiency Disorders

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Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency (PID) in adulthood and is characterized by severe reduction of immunoglobulin serum levels and impaired antibody production in response to vaccines and pathogens. Beyond the susceptibility to infections, CVID encompasses a wide spectrum of clinical manifestations related to a complex immune dysregulation that also affects liver. Although about 50% CVID patients present persistently deranged liver function, burden, and nature of liver involvement have not been systematically investigated in most cohort studies published in the last decades. Therefore, the prevalence of liver disease in CVID widely varies depending on the study design and the sampling criteria. This review seeks to summarize the evidence about the most relevant causes of liver involvement in CVID, including nodular regenerative hyperplasia (NRH), infections and malignancies. We also describe the clinical features of liver disease in some monogenic forms of PID included in the clinical spectrum of CVID as ICOS, NFKB1, NFKB2, CTLA-4, PI3Kδ pathway, ADA2, and IL21-R genetic defects. Finally, we discuss the clinical applications of the various diagnostic tools and the possible therapeutic approaches for the management of liver involvement in the context of CVID.

Keywords: primary immuno deficiency, antibody deficiency, common variable immune deficiency, liver disease, nodular regenerative hyperplasia, transient elastography, monogenic immune defects, liver transplant

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INTRODUCTION

Common variable immunodeficiency (CVID) is the most prevalent symptomatic primary immunodeficiency (PID) in adult age and is characterized by marked hypogammaglobulinemia (IgG and IgA, with or without IgM), and impaired antibody production in response to vaccines and pathogens (1, 2). CVID represents an umbrella diagnosis rather than a single disease, probably encompassing multiple genetic disorders, all leading to the failure of B-cell responses. The International Union of Immunological Societies (IUIS) Expert Primary Immunodeficiency Committee (now called Expert Committee on inborn errors of immunity – IEI) redefined in 2009 the acronym CVID as "common variable immunodeficiency disorders," thus highlighting the heterogeneity of the underlying immune defects (3). During the past 7 years, the increasing spreading of next-generation sequencing (NGS) technologies have fostered the discovery of several

genes associated with a CVID-phenotype, *via* both autosomal recessive and dominant inheritance (4, 5). This has progressively blurred the limits between humoral and combined immunodeficiency. Indeed, various genetic defects initially linked to CVID are now recognized as distinct disease entities. However, monogenic forms only account for 2–10% CVID clinical diagnosis (6). The proportion increases to 30% when considering CVID cases with criteria of monogenic form suspicion including early onset, autoimmune/inflammatory manifestations, low B lymphocytes, and/or familial history of hypogammaglobulinemia (7). The pathogenesis is more complex in the remaining cases, probably involving environment, and somatic genetic or epigenetic changes (8). Similarly, several abnormalities in immune cells' counts and function, in different combinations and in association with specific clinical features, have been described in CVID patients. Among these, the reduction of class-switched memory B cells and/or plasmablasts (9, 10), the expansion of transitional B cells and/or CD21low B cells (11, 12), the reduction of naive T cell and/or Treg cell, and the increase of peripheral blood T_{FH} cells (13, 14), are the most remarkable.

Mirroring this immunologic and genetic heterogeneity, CVID patients may experience a wide spectrum of clinical manifestations during the course of their life, including recurrent bacterial infections (mainly of gastrointestinal and respiratory tracts) and various disorders related to immune dysregulation, such as autoimmunity, granulomata, lymphoid hyperplasia, enteropathy and malignancies (15–17). The cornerstone of CVID treatment is polyvalent human IgG replacement that succeeded, over the past 4 decades, in reducing the burden of infections and improving the prognostic outcome of CVID (18–20). However, immunoglobulin replacement therapy has no proven effectiveness on immune dysregulation-related complications that consequently have become the major cause of death in CVID patients, thus demanding a more in-depth understanding of the underlying pathogenetic mechanisms (21–24).

Immune dysregulation-related complications also involve various segments of the gastrointestinal tract leading to life-threatening complications as protein-energy malnutrition, malabsorption, and gut microbial translocation (25–27). While gut or stomach involvement in CVID has been extensively described and classified by several authors, a more limited evidence is available about prevalence, pathogenesis and prognostic outcome of CVID-related liver disease (28–33). Although up to 50% of CVID patients display a persistent increase of liver enzymes associated with mild hepatomegaly, burden and nature of liver involvement have not been systematically investigated in the majority of CVID cohort studies published in the last 20 years (34, 35). Liver involvement could be defined as a disruption of liver function or portal hemodynamic and may be identified through biochemical, clinical, imaging and histologic diagnostic tools. Liver involvement in CVID is heterogeneous and may rely on immune dysregulation [i.e., nodular regenerative hyperplasia (NRH), lymphocytic infiltration, granulomatous disease], infection (i.e., viral iatrogenic hepatitis, extra-intestinal localization of *Giardia lamblia*) and malignancy (i.e., liver cancer, extra-nodal localization of lymphoid malignancies and metastatic involvement from gastrointestinal tract neoplasms). In a large United States cohort, CVID patients with liver diseases had reduced survival (HR = 2.48), compared with those without this specific complication (23). In particular, liver diseases was the fourth cause of death over a 4-decade interval, accounting for the 8.6% overall mortality. Similarly, in a recent study striking differences in mortality were observed between patients with liver disease and those without, with crude death rate of 28% and 6%, respectively (36). Prevalence information widely varies in the various cohorts (ranging from 9% to 79%) depending on the detection strategy and the sampling methodology (**Table 1**). In particular, significant heterogeneity exists between the various cohort studies with respect to the outcome variable evaluated to estimate liver impairment (i.e., liver enzyme levels, echographic features, and histopathological changes). Moreover, a large part of prevalence information is derived from cohort studies not primarily conceived to estimate liver involvement. This may result in a significant bias in prevalence data, as incomplete diagnostic assessment could have affected the detection rate of liver alterations in these studies.

Clinical, laboratory and histological signs of liver damage were present in 11.9% subjects of a large US cohort described in 1999 (37). Raised alkaline phosphatase (ALP) levels were observed in 43.5% CVID patients of a 2008 cohort study (35), while histologically proved liver disease was demonstrated in a smaller proportion of subjects in two other studies (9.1% and 9.3%, respectively) (38, 39). Our research group recently reported a liver disease prevalence of 33.8% in a cohort of 77 adult CVID patients in whom liver involvement was assessed through the measurement of liver stiffness by ultrasound-based transient elastography (TE) (40). Finally, 79% CVID patients referred to a United Kingdom Hepatology Center displayed laboratory, imaging and/or histological signs of liver disease (36).

In this review, we will summarize the evidence on epidemiology, pathogenesis, outcome, and treatment of the various forms of liver involvement in CVID (**Figure 1**). To contribute to better understand and manage CVID-associated liver disease, we will try to depict the features of liver involvement in some monogenic forms of PID included in the clinical spectrum of CVID for which specific defects in immune response pathways have been recently clarified. Finally, we will discuss the clinical applications of the various diagnostic tools employed in detection and monitoring of liver disease.

NODULAR REGENERATIVE HYPERPLASIA

Nodular regenerative hyperplasia is generally considered the most typical form of liver involvement in CVID (1). Although frequently described as a disease, NRH is actually a histopathologic picture that is thought to be the result of an intra-hepatic vasculopathy, common to various hepatic diseases, leading to both hepatocyte injury and regeneration (41, 42). This latter would determine the development of hepatocyte nodules that compress surrounding sinusoids, as well as portal and

TABLE 1 | Prevalence of liver disease in various cohorts of CVID adult patients.

Study	Year	Sample size	Study type	Prevalence (%)	Outcome variable	Clinical associations
Cunningham-Rundles et al. (37)	1999	248	Retrospective	11.9	Liver dysfunction (including viral hepatitis and primary biliary cholangitis)	NA
Ward et al. (35)	2008	108	Retrospective	43.5	Deranged liver function (i.e., increased liver enzyme levels)	Hepatomegaly, Granuloma, Cytopenias, Lymphocytic enteropathy
Malamut et al. (51)	2008	94	Retrospective	54.2	Increased liver enzyme levels, hepatomegaly and/or signs of portal hypertension	NA
Farmer et al. (38)	2018	205	Retrospective/ perspective	9.3	Histologically proved lymphoproliferative liver disease (i.e., NRH and hepatitis)	NA
Slade et al. (147)	2018	116	Cross-sectional	3	Autoimmune liver disease	NA
Azzu et al. (36)	2019	86	retrospective	79	abnormal liver function test profile OR abnormal liver imaging OR abnormal liver histology	Thrombocytopenia, splenomegaly
Crescenzi et al. (40)	2019	77	Cross-sectional	33.8	Liver fibrosis (measured as increased liver stiffness)	Polyclonal lymphoproliferation, enteropathy

central veins, thus potentially determining perisinusoidal fibrosis (**Figure 2**) (43, 44). The diagnosis of NRH is challenging due to different interpretations of the histopathologic features and the absence of either symptoms or laboratory abnormalities in most patients. Although nodularity and heterogeneous hepatic parenchyma suggestive of NRH may be detected by magnetic resonance imaging or ultrasound scan, diagnosis has to be histologically confirmed (45, 46). Recently, the revision of the histopathological definition proposed by Wanless in 1990 led to the description of NRH as focal or diffuse appearance of hepatocellular nodules less than 3 mm in diameter detected on both H&E and reticulin staining compressing peripheral sinuses, where perisinusoidal but not septal fibrosis may occur (47, 48).

Post-mortem examination studies reported a prevalence of NRH-related changes in 0.5–2.6% of general population (48, 49). NRH prevalence in CVID widely varies in the various reports, perhaps reflecting different strategies in study design and population sampling (**Table 2**). Liver biopsy is an invasive procedure that is generally performed only in the presence of clinical and laboratory clues of severe liver damage. This led to a significant underestimate in cohort studies not primarily intended to investigate liver pathology. Resnick et al. reported a NRH incidence lower than 1% over a 4-decade period, in a large perspective cohort study assessing mortality in CVID (23). On the contrary, NRH prevalence was 5% and 12% in 2 studies designed to assess the nature of liver disease in CVID patients with deranged liver function tests (35, 50). Similarly, NRH was detected in 32% patients referred to a Hepatology Center for an active follow-up (36). NRH prevalence is even higher (up to 87%) if we consider only the subset of patients undergoing liver biopsy, namely the only category where NRH diagnosis may be made or excluded with certainty (51).

Although laboratory signs of NRH may be not detectable for decades, the majority of subjects display raised ALP levels with concurrent increase in gamma-glutamyl-transpeptidase (γGT). The most common pattern of ALP derangement in CVID patients with NRH is the gradual increase over years. Otherwise, ALP levels may fluctuate or reach a peak and then return toward normal values (35). When clinical signs are present, these are the result of non-cirrhotic portal hypertension due to sinusoidal compression (50). In the most characterized cohorts of CVID patients with NRH, the most frequent clinical complications were jaundice, hepatomegaly, pruritus, ascites, and oesophageal varices, whereas decreases in neutrophil and platelet counts frequently appeared years after raising of liver enzymes (35, 50, 51). On the other hand, a subset of patients (up to 32%) may present histologically proved cirrhosis with NRH-like changes, a picture associated with higher mortality (hazard ratio = 4.2) (36). Irrespectively of the clinical course of liver disease, CVID patients with NRH are more likely to present immune-dysregulation related complications compared with those without liver involvement. Ward et al. found that NRH was significantly associated with autoimmune cytopenias, polyclonal lymphoproliferation and diffuse granulomatous disease. By contrast, no association was found with organ specific autoimmune conditions, age at onset, age at diagnosis, delay in diagnosis, and duration of immunoglobulin replacement therapy (35).

Intrasinusoidal inflammatory infiltrates represent the most common histopathological finding in CVID patients with NRH (50, 51). Immunohistochemical analysis reveals that infiltrates are mainly composed of $CD3^+$ $CD8^+$ T cells and very few B cells. Inflammatory infiltrates may co-localize with sinusoidal dilatation and/or small lobular, non-necrotizing, non-fibrosing granulomata (<50% cases). Conversely, albeit rarely,

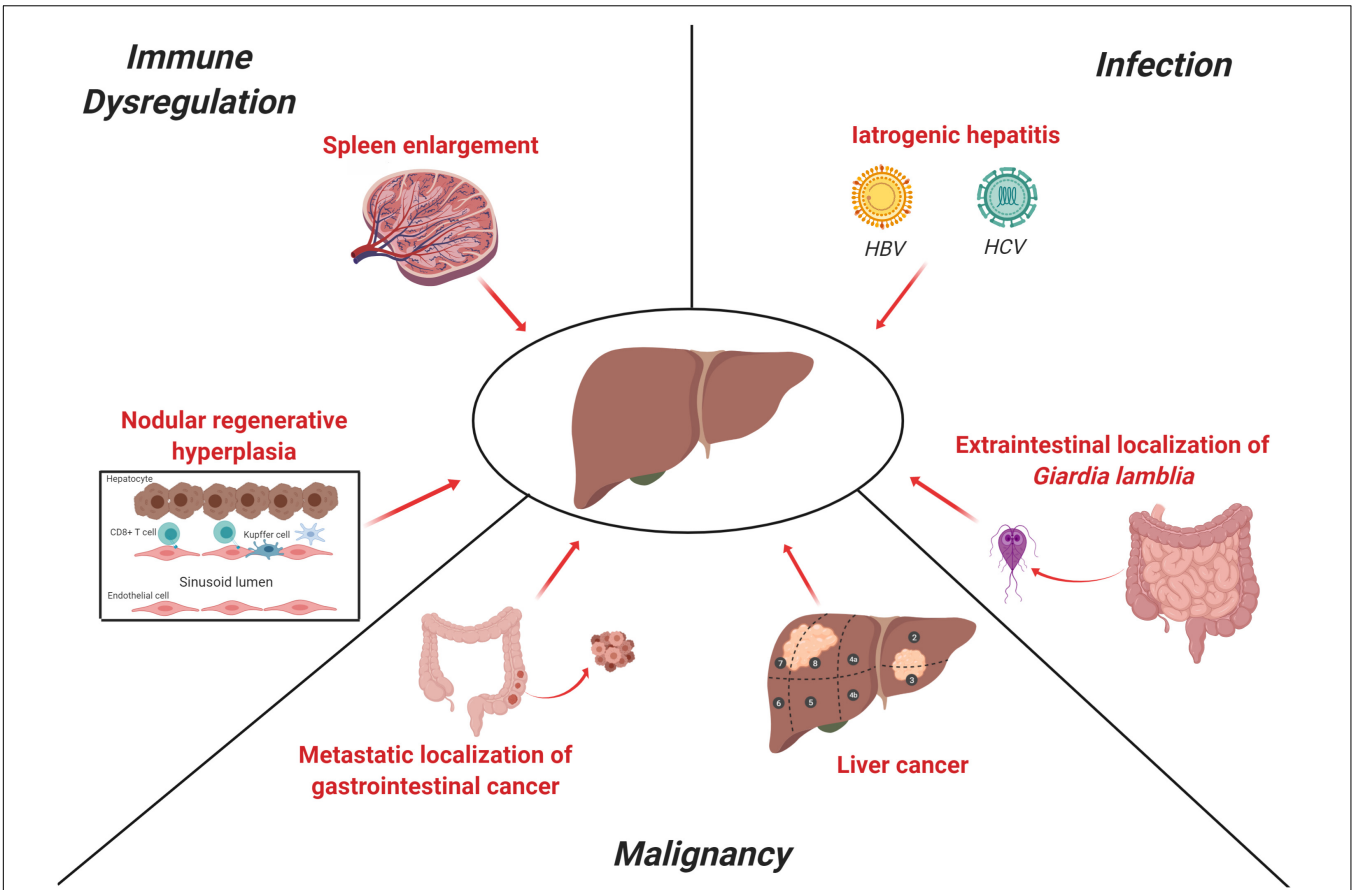

FIGURE 1 | Main causes of liver disease in CVID. The clinical spectrum of CVID includes predisposition to infections, immune-dysregulation-related manifestations (i.e., autoimmunity or lymphocytic infiltration) and malignancies. Liver involvement in CVID may rely on each of these three pathogenetic mechanisms. NRH is the most common histopathologic finding in CVID and is thought to be related, at least in part, to an auto-reactive T-cell intrasinusoidal infiltration, while a subset of patients may present a more severe portal inflammatory infiltration consistent with inferface hepatitis, as observed in autoimmune hepatitis. Splenomegaly is a common clinical feature in CVID patients and contributes to the increase of portal venous pressure, as shown in detail in **Figure 2**. In past decades, contaminated immunoglobulin preparations were a significant cause of iatrogenic viral hepatitis (i.e., HBV, HCV, CMV, and EBV), while *Giardia lamblia*, which is a common cause of chronic enteritis in CVID, may affect liver as extra-intestinal localization. Finally, liver may be target of both primary and metastatic malignancies. These latter may be the result of both gastrointestinal cancers (i.e., stomach and colon) and hematological malignancies. The figure was created with Biorender.com.

CVID patients may present liver granulomatous lesions in the context of a systemic granulomatous disease and in the absence of NRH (35). A subset of patients exhibit a more severe portal inflammatory infiltration associated with portal vein endotheliitis, bridging necrosis and periportal fibrosis, thus justifying a histological diagnosis of interface hepatitis, as observed in viral or autoimmune hepatitis (50, 51). Rather than the result of a proper autoimmune hepatitis, all these findings could represent an over-representation of the milder inflammatory infiltrate associated with perisinusoidal fibrosis, usually observed in NRH. Consistent with this hypothesis, CVID patients present interface hepatitis in the context of the nodular hepatic parenchymal pattern typical of NRH that is not described in "classical" autoimmune hepatitis (52). Besides, the diagnosis of definite autoimmune hepatitis (AIH) is very difficult to be made in CVID patients. According to the European Association for the Study of the Liver (EASL), both a histologic evidence of moderate to severe interface hepatitis and the positivity of the typical autoantibodies are required to make an AIH diagnosis (53). Indeed, as expected for a severe B-cell defect, CVID patients generally do not have autoantibodies, even in case of overt autoimmune manifestations. On the other hand, NRH *per se* is likely to represent an immune-mediated manifestation. The presence of moderate/severe inflammatory infiltrates could suggest different pathogenetic mechanisms, as well as a possible role for immunosuppressive treatments to arrest the progression of liver damage. Based on this consideration, liver biopsy would represent a pivotal tool to identify the cases of NRH associated with a more significant inflammatory infiltrate and guide the decision to start an immunosuppressive treatment.

Intrasinusoidal T lymphocytes may be involved in the pathogenesis of NRH, as supported by the frequent finding of both portal vein endotheliitis and disruption of the sinusoid lining. Indeed, a significant proportion of NRH patients display apoptotic damage of sinusoidal endothelial cells associated with the presence of $CD8^+$ cytotoxic T-cells in liver sinusoids (50, 51).

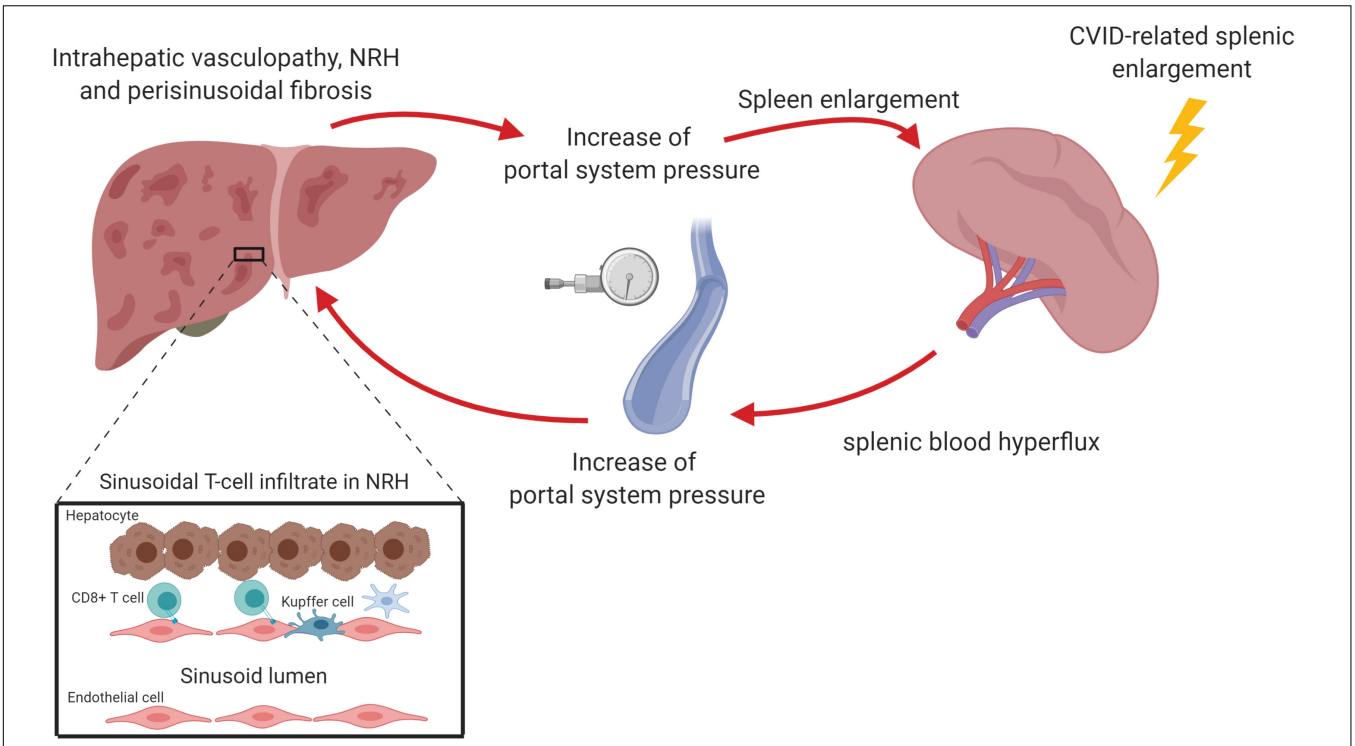

FIGURE 2 | Nodular regenerative hyperplasia (NRH) is the result of an intra-hepatic vasculopathy, leading to the development of hepatocyte nodules that compress surrounding sinusoids, potentially determining perisinusoidal fibrosis. In CVID patients, NRH is associated with a chronic cytotoxic T cell infiltration of liver sinusoidal endothelium. This may cause an alteration of the blood flow through portal system causing the reduction of liver parenchymal perfusion and the increase of portal pressure. The perturbation of portal system flow may also be the result of the hemodynamic changes related to splenomegaly, which causes an increase of splenic venous flow contributing to the increase of portal pressure. The increase of portal pressure could be in turn responsible for a further spleen enlargement. The figure was created with Biorender.com.

TABLE 2 | Prevalence of nodular regenerative Hyperplasia in various cohorts of CVID adult patients.

Study	Year	Sample size	General Prevalence	Prevalence in biopsied patients	Other findings
Ward et al. (35)	2008	108	12% (13/108)	56.5% (13/23)	Clinical association with Hepatomegaly, Granuloma, Cytopenias, Lymphocytic enteropathy
Malamut et al. (51)	2008	94	21.2% (20/94)	86.9% (20/23)	Portal hypertension in 75% of the cases Clinical association with diseases and peripheral lymphocytic abnormalities
Resnick et al. (23)	2012	473	<1% (2/473)	NA	NA
Fuss et al. (50)	2013	261	5.3% (14/261)	NA	64% of NRH patients had elevated hepatic venous pressure gradients (HVPG) consistent with portal hypertension A subset of patients either developed or presented initially with an autoimmune hepatitis-like (AIH-like) Presence of infiltrating T cells producing IFN-γ
Azzu et al. (36)	2019	86	32.5% (28/86)	41.1% (28/68)	A subset of patients had portal hypertension histological cirrhosis, associated with increase in mortality

Analysis of liver T cell receptor clonality revealed that intra-sinusoidal T cells specifically targeted sinusoidal endothelial cells. In addition to this, hepatocytes from NRH-patients exhibited overexpression (up to 100-fold) of IFN-γ mRNA compared to controls (50). These findings suggest that NRH may be the result of chronic cytotoxic T cell infiltration of the sinusoidal endothelium. This would be in turn responsible, in association with granulomata, for an alteration of the blood flow through portal system leading to the reduction of liver perfusion.

The perturbation of portal system flow may also be the result of the hemodynamic changes related to splenomegaly, a condition present in about one third of CVID patients. Pulvirenti et al. found that spleen diameter directly correlated with portal vein diameter, suggesting that an increased splenic venous flow related to splenomegaly could contribute to a condition of portal hyper-flux (54). Consistent with this, 25% patients in that cohort had ultrasound signs of portal vein enlargement, even if only 16% of them had portal

hypertension. Interestingly, when liver biopsy was performed, the authors reported micronodular transformation and lymphocytic infiltration, signs reminiscent of NRH.

Finally, some of the histopathological changes associated with NRH in CVID patients, as T-lymphocyte infiltrates and/or granulomata, may represent a response to microbial translocation. Microbial translocation is the transfer of commensal microbial products from the intestinal lumen into systemic circulation in the absence of overt bacteremia (55). Although the extent of potential damage to intestinal epithelial barrier in CVID is currently unknown, CVID patients may have increased intestinal permeability resulting from the typical CVID-related enteropathy (28). Of note, the inflammatory changes found in liver often resemble those observed in individuals with chronic inflammation of the gut. In a small group of seven CVID patients with evidence of liver inflammation, intestinal inflammation was found in five cases (56). Consistent with this hypothesis, different studies demonstrated signs of microbial translocation and microbial translocation-related immune activation in CVID patients, as elevated plasma concentration of lipopolysaccharide (LPS), soluble CD14 (sCD14), and soluble CD25 (sCD25) (57–61). However, to the best of our knowledge, no study has already addressed the possible association between impaired intestinal permeability and liver disease in CVID patients.

INFECTIONS

In the past, several cases of iatrogenic viral hepatitis C due to contaminated intravenous immunoglobulin preparations have been reported (62, 63). Several studies reported an increased mortality and morbidity in long-term follow-up of CVID patients iatrogenically infected with HCV, compared to iatrogenic viral hepatitis in general population (64–66). On the other hand, in a small study none of 18 HCV-infected patients developed severe disease nor died because of the infection (67). In more recent CVID cohort studies, a lower prevalence of viral hepatitis was reported, probably reflecting the efficient prevention of viral contamination of blood products achieved in the last three decades. Indeed, the immune defect underlying CVID would not predispose to viral infections, as also suggested by the clinical phenotyping proposed by Chapel et al. in 2008. This classification excluded viral infections, including persistent infection with enterovirus, HBV and HCV, from the clinical phenotyping, as they were not considered part of natural disease progression (68). In the same study, prevalence of hepatitis B and C among 334 CVID patients were about 1% and 6%, respectively.

Similarly, a previous cohort study from Mount Sinai Institute found an overall viral hepatitis cumulative incidence of 6.5% (37). The same research group reported a significantly lower data (1.9% and 1% for HCV and HBV, respectively) 23 years later, thus suggesting that the first cohort probably included a greater proportion of subjects who had received contaminated immunoglobulin preparations (23).

Common variable immunodeficiency patients are not particularly prone to bacterial and/or parasitic infections primarily involving the liver (69). Similarly, the finding of opportunistic or unusual pathogens, such as *Microsporidia* or *Cryptosporidia*, is rare and might suggest investigating for a combined immunodeficiency, characterized by greater degrees of T-cell dysfunction (70). Liver is a possible extra-intestinal localization of *Giardia lamblia*, which is a common cause of chronic enteritis in CVID (70, 71). Therefore, liver involvement should always be ruled out in case of *Giardia* detection from stool or duodenal samples.

MALIGNANCIES

Malignancies are one of the major causes of death in patients with CVID (72–75). Compelling evidence suggests a higher cumulative incidence of malignancy in CVID population (widely ranging from 1.5% to 25.5%), with a peak of incidence between the 4th and 6th decade of life (76). Non-Hodgkin lymphomas are the most common type of malignancy in several cohort studies (23), even though epithelial cancers are associated with a higher mortality ratio and gastric cancer has recently emerged as the leading cause of death in a large multicenter Italian study (22, 77). The pathogenetic mechanisms underlying cancer development in CVID are not completely understood. These might include impairments in various stages of B-cell maturation, primarily yielding lymphoid malignancies, chronic infections and/or low-grade inflammation, which are thought to play a pivotal role in tumor development and growth (78, 79).

In contrast with hematological and gastrointestinal mucosal malignancies, very few data are available about prevalence, distribution and outcome of liver cancers in CVID. Four cases of liver cancer were found in an Italian cohort of 455 adult patients (prevalence 0.95%), corresponding to a Standardized Incidence Ratio (SIR) of 1.9 (95% CI 0.3–5.6) in comparison to the Italian National Cancer Registry (Associazione Italiana Registro Tumori – AIRTUM) data (22). Noteworthy, all four patients died and liver cancer accounted for the 5.1% all death in the cohort. Liver cancer was the fourth cause of death for malignancy after gastric cancer, non-Hodgkin lymphoma and colorectal cancer, with a standardized mortality ratio of 2.9 (95% CI 0.1–5.9) compared to AIRTUM data. Although liver cancer is not prevalent in CVID, liver, as a secondary lymphoid organ, is a frequent extra-nodal localization of non-Hodgkin lymphoma, as well as a common metastatic target of gastrointestinal adenocarcinomas (80–85). Therefore, diagnostic protocols aiming to oncologic surveillance in CVID patients should always encompass clinical, laboratory and imaging assessment of liver to rule out its primary or secondary neoplastic involvement.

MONOGENIC FORMS OF PID IN THE CLINICAL SPECTRUM OF CVID

The striking advances in sequencing technologies have fostered the discovery of several genes associated with a CVID-like phenotype (6–8). Actually, mutations in most of them lead

TABLE 3 | Genetic, immunological and clinical features in monogenic forms of PID in the clinical spectrum of CVID with liver involvement.

Genetic defect (OMIM)	Effect on protein	Inheritance	Most frequent clinical manifestations	Most frequent Immune phenotype	Liver involvement
ICOS (86–91) (604558)	LOF	AR	Respiratory tract infections Skin infections Opportunistic infections Autoimmunity (i.e., cytopenias and arthritis)	Pan-hypogammaglobulinemia Low/absent naïve B-cells and switched-memory B cells Low T_{FH} cells Low CTLA-4 Low production of Th1/Th2/Th17 cytokines	HHV-6 hepatitis Non-infectious hepatitis (drug-induced?) Hepatomegaly
NFKB1 (92–98) (164011)	LOF (H)	AD	Respiratory tract infections Lymphadenopathy Splenomegaly, GLILD Autoimmune cytopenias Hematological malignancy	Pan-hypogammaglobulinemia Low/absent switched-memory B cells and plasmablasts Normal T-cell phenotype	Increase of liver enzymes Fibrosis and cirrhosis with Liver insufficiency
NFKB2 (99, 100) (164012)	LOF (H)	AD	Respiratory tract infections, Skin infections, Opportunistic infections Lymphocytic organ infiltration Autoimmunity ACTH-deficiency + other endocrinological abnormalities	Pan-hypogammaglobulinemia Low marginal zone and switched-memory B cells Expansion of $CD4^+$ T cell with low naïve T cells Low Treg, T_{FH} and TH17 cells	Increase of liver enzymes Lymphocytic infiltration Steatosis Autoimmune hepatitis
CTLA-4 (101–105) (123890)	LOF (H)	AD	Lymphoproliferation, Respiratory tract infections and bronchiectasis Enteropathy Autoimmune cytopenias, Atopic dermatitis Endocrinopathy Neurological disroders EBV-driven lymphomas	Pan-hypogammaglobulinemia Low $CD4^+$ T cells with normal Treg cells Low switched-memory B cells Increase of $CD21^{low}$ B cells	Unspecified liver involvement in 12% patients
LRBA (106–110) (606453)	LOF	AR	Autoimmunity cytopenias Enteropathy GLILD Lymphproliferation and lymphocytic infiltration of organs Respiratory and gastrointestinal infections Type 1 Diabetes	Pan-hypogammaglobulinemia Low switched memory B cells and plasmablasts Normal or increased double negative T cells Normal or low Treg cells	Hepatomegaly Autoimmune hepatatis Peri-portal and perisinusoidal fibrosis Granulomata
PI3Kδ pathway (111–118) (602839; 171833; 601728)	GOF of PI3Kδ (APDS1) LOF of PI3Kδ (APDS2) LOF of PTEN (APDS3)	AD	Respiratory tract infections and bronchiectasis Opportunistic and viral infections Lymphoproliferation Autoimmune cytopenia Enteropathy Neurodevelopmental delay	Low IgG and IgA Low naïve and switched-memory B cells Increase of transitional and $CD21^{low}$ B cells Low $CD4^+$ naïve T-cells Impaired T-cell response to IL-2	Increase of liver enzymes NRH Sclerosing cholangitis Cirrhosis Cryptosporidium infection
ADA2 (119–122) (607575)	LOF	AR	Recurrent infections Lymphoproliferation Polyarteritis nodosa Livedo reticularis Ischemic/hemorrhagic stroke Bone marrow aplasia Neurological impairment	Hypogammaglobulinemia Low switched-memory B cells Impaired B cell response to CD40-L and IL-21	Increase of liver enzymes NRH with portal sclerosis Vasculitis Hepatomegaly
IL-21R (123–127) (605383)	LOF	AR	Respiratory tract infections and bronchiectasis Opportunistic infections Lymphoproliferation Inflammatory skin disease	Hypogammaglobulinemia Impaired B cell response to IL-21 Variable T cell response to mitogens	Cryptosporidium infection

to more severe immune dysregulation syndromes compared to CVID, often in association with pronounced T-cell defects. Indeed, mutations affecting these genes are considered to cause separate disease entities rather than a "pure" CVID (5). Here, we discuss the monogenic forms of "CVID-like" PIDs for which liver involvement has been described, seeking to highlight the different features of liver pathology in each form, which could possibly help to drive genetic testing (**Table 3**).

ICOS

Inducible co-stimulatory (*ICOS*) deficiency was the first monogenic defect associated with CVID (86). *ICOS* biallelic mutations result in complete loss of protein expression determining low/absent memory B cells and bone marrow plasma cells (87). All *ICOS*-deficient patients present with recurrent respiratory tract infections and autoimmune manifestations (88, 89). The spectrum of disease extended to include liver involvement in 2015, when two patients presenting in early childhood with raised liver enzymes, diarrhea, colitis, and defective clearance of human herpesvirus 6 were described (90). Hepatomegaly and non-infectious hepatitis were found in 20% of a 15-patient *ICOS* deficiency cohort (91). Histological analysis revealed alcoholic steato-hepatitis in one case of non-infectious hepatitis, while pathogenesis remained unclear in the remaining cases, possibly involving drug-induced toxicity.

NFKB1

Autosomal dominant haploinsufficiency due to heterozygous loss-of-function mutations in nuclear factor kB subunit 1 (*NFKB1*) causes a progressive impairment in the development of immunoglobulin-producing B cells and is now recognized as the most common monogenic cause of CVID (92, 93). Massive lymphadenopathy, splenomegaly and autoimmune cytopenias are the main clinical features of *NFKB1* LOF (94). Liver involvement was described in 37.5% (6/16) patients in a European population study: three patients had persistently raised liver enzymes and three developed liver failure (95). Histologic assessment of liver disease was performed in three patients, showing fibrosis and cirrhosis with no evidence of autoimmune or granulomatous disease. Consistent with this finding, mouse models have suggested a non-immune role for NF-kappa B signaling in patients with liver failure (96). Multiple liver hemangioma and hepatomegaly associated with EBV-driven lymphoproliferation were described by two previous reports (97, 98).

NFKB2

The clinical phenotype of nuclear factor kB subunit 2 (*NFKB2*) haploinsufficiency is characterized by early-onset antibody deficiency, autoimmunity, lymphocytic organ infiltration and possibly ACTH-deficiency (99). Liver abnormalities reported in literature are parenchymal lymphocytic infiltration (2 patients), mild hepatopathy with elevation of liver enzymes, liver steatosis and histologically proved autoimmune hepatitis (one patient each) (100).

CTLA-4

Cytotoxic T-lymphocyte antigen 4 (*CTLA4*) is an essential negative immune regulator acting in the suppression of T-cell proliferation and differentiation mediated by regulatory (Treg) cells (101, 102). Heterozygous germline mutations in *CTLA4* cause an immune dysregulation and immunodeficiency syndrome including hypogammaglobulinemia, lymphoproliferation, recurrent respiratory infections and bronchiectasis, enteropathy, autoimmune cytopenias, atopic dermatitis, endocrinopathy, and neurological features (103, 104). The largest multicenter cohort, including 90 affected subjects within 133 *CTLA4* mutation carriers, reports a prevalence of 12% (11/90) of unspecified liver involvement (105). Liver cirrhosis of unknown cause was identified in one patient, while one mutation carrier died for acute liver failure after many years of gastrointestinal disease.

LRBA

The lipopolysaccharide-responsive and beige-like anchor (*LRBA*) protein deficiency is caused by loss of protein expression, which can be the result of either homozygous or compound heterozygous mutations in *LRBA* (106). *LRBA* plays a pivotal role in *CTLA-4* surface expression, by rescuing endosomal *CTLA-4* from lysosomal degradation. Clinical manifestations of *LRBA* deficiency include early-onset hypogammaglobulinemia, autoimmune manifestations, IBD and recurrent infections (107). The largest cohort study, describing clinical features of *LRBA*-deficiency in 22 subjects, reports hepatomegaly in 24% patients, with three subjects diagnosed with autoimmune hepatitis (108). Histopathological features of liver disease in *LRBA* deficiency have been investigated in a small number of case series, which described lymphocytic (T cell) infiltrates suggestive of autoimmune hepatitis and/or portal and periportal fibrosis associated with bridging cirrhosis and/or granulomata (106, 109, 110).

PI3Kδ PATHWAY

Germline mutations leading to hyperactivation of the phosphoinositide 3–kinase δ (*PI3K*δ) pathway cause activated phosphoinositide 3–kinase δ syndrome (APDS) (111). This may be the result of heterozygous gain-of-function mutations in the calalytic subunit of *PI3K*δ – *PIK3CD* (APDS1), heterozygous loss-of-function mutations in the regulatory subunit of *PI3K*δ – *PIK3R1* (APDS2), or loss-of-function mutations in phosphatase and tensin homolog – *PTEN* (APDS3) (112, 113). The most frequent clinical manifestations of APDS are recurrent bacterial and viral infections and non-malignant lymphoproliferation (114). This latter also includes hepatomegaly, typically in association with lymphadenopathy and splenomegaly. In a large series of APDS patients, raised liver enzymes were observed in 27% (9/33) subjects. NRH was the most frequent histological diagnosis (4/5 patients undergoing liver biopsy) and was associated with mildly increased portal pressure, even though clinical signs of portal hypertension were only present in one patient (115). The high prevalence of NRH has possible therapeutic implications, since NRH is known to lead to poor outcome after hematopoietic stem cell transplant (HSCT), which

represents the only curative approach to APDS (116). Therefore, the detection of NRH before HSCT may influence the choice of myeloablative preconditioning. Finally, rare cases of cirrhosis and primary sclerosing cholangitis have been reported in APDS cohort studies, while Cryptosporidium species has been isolated in only two cases (117, 118).

ADA2

Loss-of-function mutations in adenosine deaminase type 2 (*ADA2*) result in an autosomal recessive disease characterized by a heterogeneous clinical picture, probably mirroring the pleiotropic effects of this enzyme (119). Clinical manifestations of deficiency of *ADA2* (DADA2) include hypogammaglobulinemia, recurrent infections, bone marrow aplasia, pure red cell aplasia, neutropenia, liver disease, neurological impairments, and vasculopathy of small- and medium-sized arteries (120, 121). Liver biopsies from DADA2 patients revealed vascular changes characterized by compromised endothelial integrity, endothelial cellular activation and inflammation (120). Elevated liver enzymes and hepatosplenomegaly are the most common liver-related clinical signs (120–122). Histopathologic assessment frequently shows NRH and/or hepatoportal sclerosis, which could potentially lead to portal hypertension and end-stage liver disease (120).

IL21R

Biallelic loss-of-function mutations in IL21 receptor (*IL21R*) cause a severe syndrome characterized by respiratory tract infections, inflammatory complications and/or opportunistic infections, with elevated mortality in childhood (123). To the best of our knowledge, four *IL21R*-deficient patients with Cryptosporidium-related liver disease have been described (124–126). Of note, one of the first two index patients underwent liver transplantation (LT) before both the underlying PID and the Cryptosporidium infection had been recognized (124). He died shortly after the procedure due to multiorgan failure. Although no clinical association between *IL21R* deficiency and liver malignancy has been described in humans, an interesting mice model demonstrated that *IL21R* signaling deficiency might promote hepatocellular carcinoma (HCC) growth. Interestingly, Zheng et al. reported that *IL21R* deletion reduced T cells infiltration, activation and functions while increased the infiltration of myeloid-derived suppressor cells that enhanced HCC growth (127). If confirmed in human studies, this finding could affect long-term follow-up strategies of liver involvement in *IL21R*-deficient patients.

DIAGNOSTIC WORK-UP

The laboratory panel to assess liver impairment in CVID includes full blood count, liver function tests – LFTs (i.e., AST, ALT, ALP, γGT, total protein, and albumin) and clotting profile (i.e., INR, APTT, fibrinogen). Given the heterogeneity of liver disease, as well as the number of drugs (notably immunosuppressant) and the wide range of non-primarily hepatic complications that may possibly affect liver function in the context of CVID, we believe that this profile should be repeated every 4–6 months, also in asymptomatic patients (15–17, 128). In addition, we perform a wide screening for hepatitis viruses based on nucleic acids detection methods, at the time of diagnosis and at 1-year intervals, due to the virtual risk of viral contamination of immunoglobulin preparations (62–64). Actually, this timing reflect our own clinical practice as no specific guidelines or clinical consensus have been defined. ALP is the most commonly elevated liver enzyme in CVID and its increase is up to twofold above the upper limit on overage (34, 50). Ward et al. identified three distinct patterns of ALP derangement in CVID patients with abnormal LFTs, consisting in progressive elevation, fluctuating increases and transient increase (35). In a cohort of CVID patients with NRH, ALP raise was first observed 6–10 years after the time of CVID diagnosis, while the increase in ALT/AST ratio occurred over the same period but at a lesser degree (50). Noteworthy, elevation of ALP may also be caused by osteomalacia as a result of enteropathy or granulomatous disease, which are common complications in CVID (34, 35).

Ultrasonography, computed tomography scan (CT), or magnetic resonance imaging (MRI) may be employed to detect structural changes (as signs of NRH, cirrhosis and/or portal hypertension), estimate hepatomegaly and/or splenomegaly, and rule out primary or secondary malignant involvement (34). Due to low costs, wide availability, and non-invasiveness, we suggest performing ultrasonography with Doppler-evaluation as first-line liver imaging in all CVID patients, while CT and MRI may be prescribed, even at the suggestion of the Radiologist or the Hepatologist, to better characterize abnormalities detected by US.

Results of CT and MRI scans revealed portal vein dilatation and collateral vessel formation in 50% CVID patients with NRH described by Fuss et al. (50), while abnormal liver imaging was present in 77% of CVID patients started to an active hepatology follow-up reported by a more recent United Kingdom cohort study (36). On the other hand, histopathological changes consistent with NRH were found in a subset of patients with normal liver imaging, who had undergone liver biopsy because of abnormal LFTs. This suggests that liver biopsy should be considered in all patients with persistently abnormal LFTs (36).

In the last decade, ultrasound-based TE has been increasingly used to improve the detection of the progression of liver damage in the context of chronic HCV-disease (129). TE allows estimating the degree of liver fibrosis through the assessment of liver stiffness and depends on vibration generating machine to apply vibrations to the liver and then obtain the propagation velocity of shear wave (130). We recently investigated liver involvement in a cohort of CVID adult patients by means of ultrasound based TE, finding that 33.8% patients presented increased liver stiffness values ranging from moderate fibrosis to cirrhosis (40). Interestingly, TE values were correlated with ALP and γGT values, spleen longitudinal diameter and peripheral blood counts. Moreover, liver stiffness was higher in patients with polyclonal lymphoproliferation and/or enteropathy, and subjects harboring both these complication showed a significantly

increased risk (OR: 7.14) of having increased TE values. Therefore, given its non-invasive nature, the limited costs and the crucial information provided, we suggest repeating ultrasound-based TE, as well as canonical ultrasound scan with Doppler evaluation, every 12 months, also in asymptomatic CVID patients. On the other hand, although TE assessment allow to reliably estimating fibrotic changes of liver parenchyma, it does not provide information about the extent and the trend of stiffness variations related to the differet underlying pathogenetic process (i.e., inflammation, granulomatous disease, and lymphocytic infiltration). Further studies, evaluating the concordance between stiffness values and liver histological changes, are required to assess the role of elastography in the evaluation and management of liver involvement CVID.

While the spreading of TE and the systematic use of the various imaging techniques may determine a reduced overall need for liver biopsy, histological analysis of the hepatic parenchyma remains the only tool to ascertain the etiopathogenetic nature of liver damage and confidently estimate its outcome (131). On the other hand, liver biopsy is an invasive procedure, associated with an estimated morbidity and mortality rate in general population of 3% and 0.01%, respectively, with bleeding being the most relevant cause (132). In the context of CVID, this procedure may be theoretically burdened by an additional infectious risk due to the underlying immune defect. Moreover, liver biopsy provides only a very small part of the whole organ, which could be not representative for the degree of the pathological status of the remaining parenchyma, due to the heterogeneity usually observed in liver injury distribution (133). In general, indications for liver biopsy fall into two groups: establishing a diagnosis (including the assessment of the predominant cause of liver injury if more than one is present) and staging/grading liver damage (134). Indeed, in both cases the result of histological assessment may modify the therapeutic management, offering the patient personalized therapeutic options. We suggest that liver biopsy should be considered for CVID patients with a significant (more than twofold the upper limit of the range) unexplained increase of one or more liver enzymes, lasting more than 6 months. The association with pathological liver stiffness values and/or imaging findings of uncertain interpretation strengthens this recommendation. However, we believe that the decision to perform a biopsy and its timing should rely on both the pathological processes being suspected and the possibility of a potential therapeutic intervention.

THERAPEUTIC PERSPECTIVES: LIVER TRANSPLANTATION AND HSCT

Irrespectively of the ethiopathogenesis and despite the adequate treatment of complications (i.e., portal hypertension, jaundice, and oesophageal varices), chronic liver inflammation may cause a progressive disruption of liver function that is not improved by immunoglobulin replacement therapy. Moreover, there are no available medical treatments to arrest the histopathologic progression of NRH, which is the most common form of liver involvement in CVID and is complicated, in a subset of patients, by portal hypertension or overt hepatic cirrhosis with end-stage liver disease (44–47). In these cases, LT is the only therapeutic approach that has the potential to provide a long-term survival advantage (135). According to the European Association for the Study of the Liver (EASL), LT should be considered in any patient with end-stage liver disease, in whom the LT would extend life expectancy beyond what the natural history of underlying liver disease would predict or in whom LT is likely to improve the quality of life (136).

On the other hand, the theoretical increase of infectious and neoplastic risk related to the long-term concomitant immunosuppressive therapy has historically determined a reluctance to perform LT in CVID patients. In the last decade, a growing number of reports described the outcome of LT performed in adult and pediatric CVID patients with viral hepatitis or NRH (137–143).

A retrospective Norwegian cohort study reported five CVID patients transplanted over a 20-year period (137). The first patient, transplanted in 1993 for HCV-related disease, died because of sepsis combined with a debilitating *Cryptosporidium parvum* infection and cytomegalovirus pneumonitis, whereas, the 4 patients transplanted between 2009 and 2013 for definite or probable NRH, were alive at the time of publication, with a median survival of 5 years. This different outcome is likely to be related to the changes in immunosuppressive drug regimens from the 1990s to 2009–2013, which consist in the decrease of the glucocorticoid doses. More recently, Azzu et al. described four CVID patients undergoing LT for end-stage liver failure, in whom histological examination revealed NRH-like changes (138). In three subjects out of four, post-transplant course was complicated by multiple infectious complications (including *Pneumocystis jiroveci* pneumonia, toxoplasmosis, neuro-aspergillosis, and CMV proctitis), early recurrent disease, and in one patient, death due to malignancy within 3 years of transplantation. Noteworthy, histological examination showed NRH changes and cholestasis in all three patients undergoing post-transplant biopsy, as already previously described in non-immunodeficiency subjects (144). After a revision of literature data, including 18 patients, the authors found that CVID patients undergoing LT had a higher mortality compared to LT in general population, with only 55% subjects alive after 3-5 years of post-transplant follow-up (138). Moreover, CVID patients undergoing LT due to CVID-related liver disease (namely NRH) exhibited a worse 5-year survival compared to CVID patients who received LT for any cause (mainly chronic viral hepatitis) (138). This probably reflects the fact that the latter subset of patients presented a lower incidence of immune dysregulation-related complications, which are associated with worse long-term survival and higher risk of recurrent of disease in the graft. In this subset of patients, there could be a theoretical benefit of combined hematopoietic stem cell and LT.

Hematopoietic stem cell transplantation (HSCT) could theoretically prevent the development of liver disease or arrest progression in subjects with established liver disease, with a significant improvement of long-term outcome. HSCT is the standard of care of a broad group of severe combined

primary immunodeficiencies primarily affecting T-cell functions, as well as of other complex primary immunodeficiencies (i.e., chronic granulomatous disease, Hyper-IgE syndrome, Wiskott-Aldrich syndrome, etc.) (145). The growing evidence of both T-cell defects and poor outcome in the subset of patients with marked immune dysregulation, have progressively fostered the interest in HSCT for the treatment of CVID. In the largest multicenter study collecting data of CVID patients undergoing HSCT, overall survival rate was 48% after 2 years, with immune dysregulation (i.e., autoimmune cytopenias, enteropathy, generalized granulomatous disease) and hematological malignancies being the major indications to transplantation (146). The major causes of death were treatment-refractory graft-versus host disease (GvHD), poor immune reconstitution and infectious complications. On the other hand, IgRT was stopped in 50% and the condition constituting the indication for HSCT resolved in 92% of surviving patients, thus suggesting that this therapeutic approach could be beneficial in selected patients. Indeed, the definition of criteria for both patient selection and transplantation timing, as well as the refinement of the procedure protocol, are urgently needed to improve the outcome of CVID patients undergoing HSCT.

CONCLUSION

Although more than 50% CVID patients exhibit clinical or biochemical signs of liver derangement, burden and nature of liver involvement have not been systematically investigated by the major part of CVID cohort studies published in last decades. This lack of evidence lead to the absence of indications or guidelines concerning diagnosis, investigation and management of CVID-associated liver disease in clinical practice. Moreover, the striking advances in sequencing technologies has fostered the discovery of several genes associated with monogenic CVID disorders for which specific liver alterations have been described. We sought to provide a comprehensive overview of both the different causes of liver involvement in CVID and the various monogenic defects associated with liver disease, in order to facilitate the Clinical Immunologist in the diagnostic and therapeutic approaches. The clinical spectrum of CVID includes predisposition to infections, immune-dysregulation-related manifestations (i.e., autoimmunity or lymphocytic infiltration) and malignancies. Liver involvement in CVID may rely on each of these three pathogenetic mechanisms NRH is the most common liver histopathological change observed in CVID patients and is thought to be the result of an intra-hepatic vasculopathy, leading to the development of hepatocyte nodules that compress surrounding sinusoids, potentially determining perisinusoidal fibrosis. Therefore, NRH has the potential to determine a significant alteration of the blood flow through portal system, thus promoting the development of portal hypertension. Infections could either primarily (as in the case of iatrogenic viral hepatitis due to contaminated immunoglobulin preparations in past decades) or secondarily (extra-intestinal localization of parasites) affect liver. Similarly, liver may be target of both primary and metastatic malignancies. Given the heterogeneity of liver disease and the possible impact on long term outcome, each CVID patient should be screened for a possible liver impairment through biochemical (i.e., AST, ALT, ALP, γGT, and total protein and albumin) and morphological (i.e., ultrasonography, TE, and eventually CT or MRI) assessments that should be performed at regular intervals. These diagnostic tools may help to timely identify liver involvement, monitor its progression and select patients eligible to liver biopsy. Despite early detection and adequate treatment of complications, chronic liver damage may progress toward an end-stage disease. In these cases, LT and hematopoietic stem cell transplantation are the only therapeutic approaches that have the potential to provide a long-term survival advantage, even though serious warnings still subsist about the outcome of these procedures in CVID patients. Indeed, compelling evidence concerning the applications of these therapeutic options are urgently needed.

AUTHOR CONTRIBUTIONS

AP, LC, and GS conceived the work and selected the data sources. AP and LC wrote the manuscript and realized the figures. All authors revised the data sources and manuscript text.

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REVIEW
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Bronchiectasis in Primary Antibody Deficiencies: A Multidisciplinary Approach

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Bronchiectasis, the presence of bronchial wall thickening with airway dilatation, is a particularly challenging complication of primary antibody deficiencies. While susceptibility to infections may be the primary factor leading to the development of bronchiectasis in these patients, the condition may develop in the absence of known infections. Once bronchiectasis is present, the lungs are subject to a progressive cycle involving both infectious and non-infectious factors. If bronchiectasis is not identified or not managed appropriately, the cycle proceeds unchecked and yields advanced and permanent lung damage. Severe symptoms may limit exercise tolerance, require frequent hospitalizations, profoundly impair quality of life (QOL), and lead to early death. This review article focuses on the appropriate identification and management of bronchiectasis in patients with primary antibody deficiencies. The underlying immune deficiency and the bronchiectasis need to be treated from combined immunology and pulmonary perspectives, reflected in this review by experts from both fields. An aggressive multidisciplinary approach may reduce exacerbations and slow the progression of permanent lung damage.

Keywords: bronchiectasis, antibody deficiencies, primary immunodeficencies (PID), immunoglobulin replacement therapy (IgRT), pulmonary therapy, non-infectious complications

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BACKGROUND

Primary antibody deficiencies are the most common inherited form of primary immunodeficiency (1). Disorders within this group, all of which involve impaired function of the B cell compartment, encompass a wide and rapidly growing array of underlying genetic causes. An intrinsic B cell defect may be the primary cause of the disorder (predominantly antibody deficiencies), or B cells may be unable to function optimally secondarily to numerous other forms of immune defects. Patients suffer from chronic and recurrent bacterial sinopulmonary infections with common respiratory pathogens such as non-typeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae* (2). Infections with atypical bacteria (*Mycoplasma* and *Ureaplasma* species) as well as increased frequency and severity of common viral respiratory infections may occur (2). Patients should undergo laboratory workup to evaluate for a potential antibody deficiency when they experience sinopulmonary infections with unusual frequency, duration, or severity (3). The most profound example of a predominantly antibody deficiency is agammaglobulinemia, which has undetectable B cells (4). While X-linked agammaglobulinemia (XLA), due to absent Bruton's tyrosine kinase

(BTK) was initially recognized to cause arrested B cell development, there are now at least 12 known molecular defects which lead to agammaglobulinemia (4). The most common clinically relevant predominantly antibody deficiency is common variable immune deficiency (CVID). CVID can be defined as the following: Low IgG and at least one other low isotype (IgA or IgM) with poor response to vaccines (5). CVID is not diagnosed in infants and toddlers, as young patients may have a transient antibody defect. Much debate has centered on the definition and diagnostic criteria of CVID, stemming primarily from the rapidly increasing knowledge on numerous underlying molecular causes of a similar immunologic phenotype. The most recent International Union of Immunological Societies (IUIS) expert committee report lists 20 molecular defects which have been documented to cause an antibody deficiency consistent with CVID (4). If a genetic cause for the antibody deficiency is identified, the patient is diagnosed with the specific genetic immune defect, not CVID (5). While autoimmunity and immune dysregulation may be seen in many forms of immune deficiency, these problems are particularly common in patients with CVID (25–30% of patients) (2). Up to 94% of patients with CVID have some degree of detectable lung abnormalities by chest computed tomography (CT) (6). The diagnosis of bronchiectasis is made in ∼23% of patients with CVID, according to the European Society for Immunodeficiencies Registry, with a large variation in prevalence (0–66%) between centers (7). Because CVID accounts for up to one-third of all primary immunodeficiencies (8), much of the discussion throughout this manuscript focuses on this immune deficiency. It is essential, however, to realize that any clinically relevant antibody deficiency may lead to bronchiectasis. The IUIS regularly updates an extensive classification on antibody deficiencies which serves as an excellent resource (4).

Bronchiectasis is a disorder of airway architecture associated with chronic inflammation, manifesting usually as irreversible dilatation of the bronchi (9). The bronchial dilatation causes difficulty clearing bacteria and mucus from the airway, contributing to persistent infection and inflammation. Airway damage may proceed in a vicious inflammatory cycle (**Figure 1**). Patients with antibody deficiencies are at high risk for bronchiectasis not only because they cannot defend the lungs effectively against infections, but also because they may have a dysregulated inflammatory response.

The lens through which bronchiectasis is viewed has changed over time. Laënnec first described bronchiectasis in 1819 (10), only 3 years after he invented the stethoscope. At that time, antibiotics were not available and severe chest infections such as tuberculosis were common. Such chest infections often resulted in bronchiectasis, if the patient survived. In the modern antibiotic era, bronchiectasis occurs almost exclusively in the setting of an underlying host defect. Primary immunodeficiency accounts for 12–34% of non-cystic fibrosis (CF) bronchiectasis (11). While up to half of non-CF bronchiectasis cases remain idiopathic, the low incidence of bronchiectasis in developed countries suggests that such cases have a yet to be identified infection or an underlying defect in mechanisms involving the immune system, local defenses, or mucociliary clearance. Bronchiectasis is the result of multiple pathophysiological processes which occur in patients with a diverse list of underlying disorders. The heterogeneous nature of this disorder has led some to argue

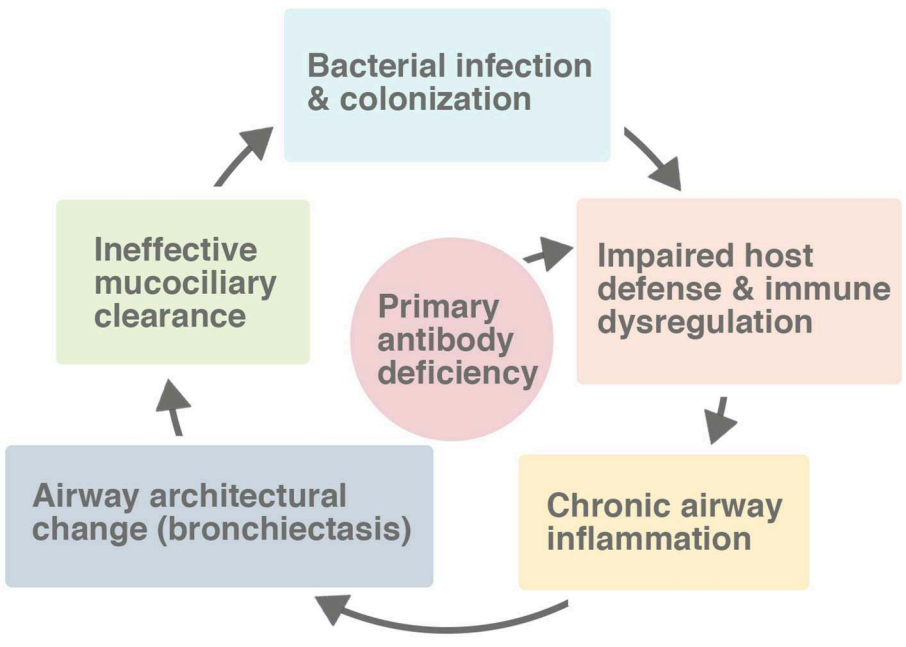

FIGURE 1 | The vicious cycle leading to bronchiectasis in the setting of a primary antibody deficiency. While the host defect of the immune system impairs the response to pulmonary infections, the defect also directly causes immune dysregulation and a chronic inflammatory state in many patients. Many factors, including secondarily impaired mucociliary clearance, contribute to the process of progressive lung damage.

that bronchiectasis is among the most complex and challenging disorders in respiratory medicine (12).

Although two centuries have passed since bronchiectasis was first described, there remains much to be learned. The pathophysiologic processes leading to bronchiectasis remain poorly understood, and patients may often experience significant delays in diagnosis. In children, the early signs of bronchiectasis are often misdiagnosed as asthma (13). Early, optimal treatment, arrest of progression, and, ultimately, reversal of bronchiectasis, are goals which have not been widely met (14). Specifically, the clinical approach to bronchiectasis in the setting of antibody deficiencies lacks sufficient studies and clinical guidelines. The goal of this review is to combine and summarize currently available evidence with expert opinion from both the pulmonology and immunology fields regarding the comprehensive, multifaceted approach to bronchiectasis diagnosis and treatment in patients with antibody deficiencies.

THE IMPACT: MORBIDITY, MORTALITY, AND QUALITY OF LIFE

For adult patients with bronchiectasis, age-adjusted mortality is more than twice that of the general population (15). Predictors of mortality include old age, low forced expiratory volume in one second (FEV_1), low body mass index (BMI), history of hospitalizations, and frequent exacerbations (12). Progressive airway damage can lead to respiratory failure and death (16). Persistent daily respiratory symptoms, fatigue, frequent and prolonged hospitalizations, and the mostly incurable nature of the disorder may negatively impact patient quality of life (QOL) and places significant stress on the patient and caregivers (12).

PATHOPHYSIOLOGY: THE IMMUNE SYSTEM, INFLAMMATION, AND THE LUNG

In patients with antibody deficiencies, the lung is often considered the organ most susceptible to damaging infections, as it presents a vast mucosal surface to the environment. The surface area of the adult human lung is approximately equal to one-half of a tennis court and is exposed to 10,000 L of environmental air every day (17). Considering that the lining of the lung is composed of a membrane so thin that it readily allows gas exchange, it is not surprising that patients with antibody defects may fail to fully defend the lung from infections. Infections often launch the vicious cycle of progressive lung damage which culminates into bronchiectasis (**Figure 1**). Globally, severe lower respiratory tract infections early in life are the leading cause of bronchiectasis, with measles and tuberculosis accounting for 25% of post-infectious pediatric bronchiectasis (18).

Mucociliary function may become secondarily impaired following the prolonged and recurrent infections experienced by patients with antibody deficiencies (12) (**Figure 2**). It is likely that weakened mucociliary function and biofilm formation play a significant role in driving chronic inflammation (12). Both local and systemic inflammation is increased in the setting of bronchiectasis and may persist even in the absence of infection (19). Within this inflammatory response, phagocytes and eosinophils have been identified as contributors to ongoing damage. Phagocyte recruitment is promoted by epithelial cells which release pro-inflammatory cytokines (20). Neutrophils and eosinophils contribute to airway inflammation and damage directly via degranulation (21). Although patients often have an increased number of airway macrophages, the ability of these cells to clear apoptotic neutrophils from the airway, as well as phagocytosis of NTHi, is impaired (22). Exhaled hydrogen peroxide (H_2O_2) has been used as a marker for activated neutrophils (23). Increased levels of H_2O_2 correlate with increased neutrophil burden, and worsened disease severity and lung function (23). Neutrophil elastase (NE), a pro-inflammatory serine protease released from azurophilic granules, may also be used as a marker of disease severity (24). NE slows ciliary beat

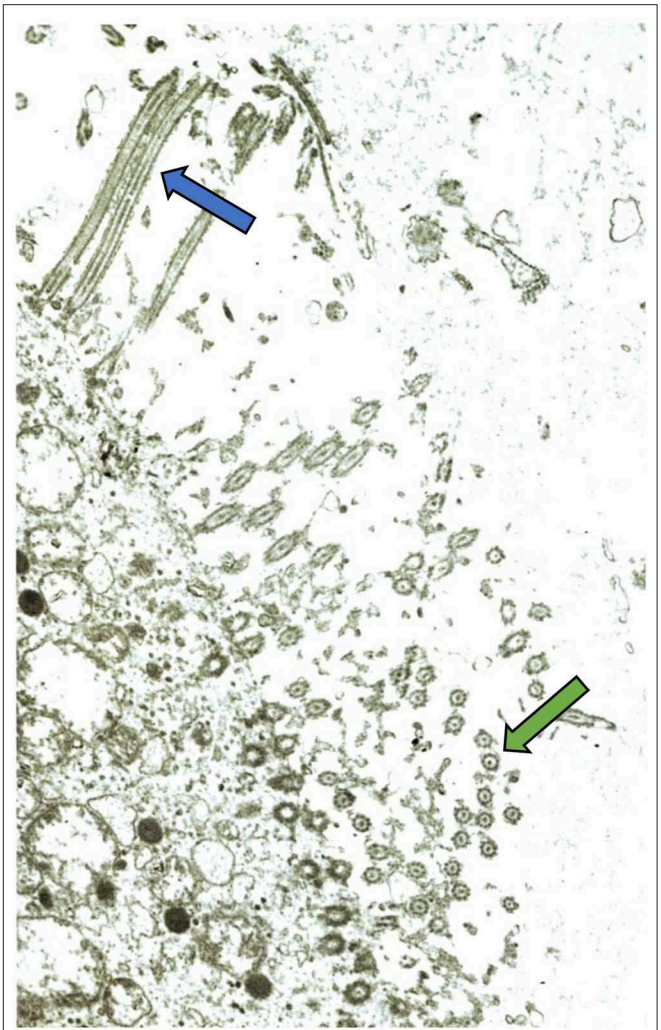

FIGURE 2 | Electron micrograph of bronchial mucosa from a child with chronic chest symptoms. Despite normal intrinsic ciliary structure, a general paucity of cilia is demonstrated. This is a common finding on ciliary biopsy in the setting of recurrent respiratory infections. Green arrow indicates cilia seen in cross-section. Blue arrow indicates cilia seen lengthwise. Image courtesy Matthew Stark, MD, Pathologist, Children's Hospital New Orleans.

frequency and increases mucus secretion, further contributing to the progression of disease (24). In the normal lung, NE is inhibited by anti-proteases such as alpha-1-antitrypsin (24). In bronchiectasis, release of NE overwhelms the anti-protease defense, leading to detectable levels of NE proteolytic activity in sputum (24). Linking pathogenesis with targeted therapies, NE is the focus of current trials for specific therapeutic agents in the treatment of bronchiectasis (25).

Bronchiectasis does not develop in all patients following lower respiratory tract infections, highlighting the essential concept that bronchiectasis encompasses many structural, functional, immunologic, and inflammatory processes. Interestingly, the neonatal Fc receptor (FcRn) may play a role. While this receptor is responsible for transplacental transfer of IgG from mother to fetus during pregnancy, it also plays a crucial role in IgG homeostasis throughout life (26). It is expressed on epithelial cells as well as vascular endothelial cells throughout the body (26). This transcytosis receptor maintains IgG in the circulation by facilitating the recycling of IgG, and is an important sensor of luminal infections (26, 27). In patients with CVID and bronchiectasis, lower FcRn mRNA expression was found to correlate with increased severity of bronchiectasis and a higher rate of IgG decline following intravenous immunoglobulin (IVIG) infusion (27). Studies of bronchiectasis in general, summarized below, have demonstrated that a disordered inflammatory response is an important variable, as is an impaired T cell response to pathogens. These are problems which are relatively common in some forms of antibody deficiencies such as CVID. A large observational study of CVID patients, for instance, demonstrated that 20% of CVID patients had low circulating CD4+ T cells, 40% had subnormal T cell proliferation response to one or more mitogens, and 22% demonstrated clinical autoimmune disease (28).

Cell-mediated immune response to NTHi, the most common respiratory pathogen in patients with bronchiectasis, has been found to be impaired in children with chronic suppurative lung disease (CSLD), which is considered a prelude to bronchiectasis. NTHi is unique in that it produces human IgA proteases which may contribute to the invasion of respiratory epithelium (18). It is also capable of intracellular survival in host macrophages and respiratory epithelial cells (29). Pizzutto et al. measured a panel of pro-inflammatory cytokines, antimicrobial proteins, and cellular and clinical factors associated with airway inflammation in 70 children with CSLD (30). IFN-gamma was measured in PBMCs challenged *in vitro* with live NTHi. On multivariate regression, NTHi-specific IFN-gamma production was negatively associated with the BAL concentrations of IL-6 and IL-1beta. Therefore, in these patients, increased local airway inflammation was associated with impaired cell-mediated immune response to NTHi (30). In addition, in a study of adults with bronchiectasis, cytotoxic T cell demonstrated less IFN-gamma production *in vitro* to NTHi compared to controls (29). Collectively, this evidence suggests that an impaired cell-mediated immune response may lead to increased susceptibility to NTHi and contribute to the pathogenesis of CSLD, the precursor of bronchiectasis.

The immunologic milieu involved in the development of bronchiectasis specifically among patients with antibody deficiencies has not been well studied. It is possible that the previously described inflammatory processes in the lung are amplified, at least in some patients who have antibody deficiencies associated with immune dysregulation, such as CVID. In patients with CVID, bronchiectasis has been known to progress in the absence of obvious infections (31). The propensity toward autoimmunity and chronic inflammation in CVID is a proposed factor (32, 33). Insights which link the concepts of autoimmunity, chronic inflammation, susceptibility to lung infections, and the importance of neutrophils may suggest another clue regarding the pathogenesis of bronchiectasis (34). Specifically, the endotoxin-binding bactericidal/permeability-increasing protein (BPI) is present in leukocyte granules (35). It has antibacterial and anti-endotoxin properties (35). The presence of anti-BPI autoantibodies is associated with persistence of *Pseudomonas aeruginosa* and worse lung function in children with CF (34). In addition, anti-BPI auto-reactivity has been found to be strongly associated with the presence of anti-*P. aeruginosa* antibodies in two bronchiectasis cohorts in North America, suggesting that the breaking of tolerance to BPI is mediated through an association with *P. aeruginosa* infection (36).

Specific clinical and laboratory features have been found to correlate with the development of bronchiectasis in patients with antibody deficiencies as described in the "Identifying Bronchiectasis" section of this article. While such information may be helpful in knowing when to suspect bronchiectasis in a patient with an antibody deficiency, it sheds little insight into the underlying cause. Currently, our understanding of pathogenesis is limited mostly to extrapolation of studies examining the immune response in patients with bronchiectasis in general. As demonstrated above, there is a need for additional studies in patients with antibody deficiencies such as CVID, who have a predisposition to develop autoantibodies, yet cannot form protective antibodies appropriately. Understanding how underlying systemic immune dysregulation contributes to the development of bronchiectasis in patients with antibody deficiencies holds important potential implications for both prognosis and treatment.

IDENTIFYING BRONCHIECTASIS IN PATIENTS WITH PRIMARY ANTIBODY DEFICIENCIES

Clinically, bronchiectasis presents with persistent or recurrent productive cough, airway infections, and pulmonary exacerbations (37). Although young children often do not expectorate, a wet-sounding cough is typically present and can be helpful in identifying patients suspected of having bronchiectasis (37). Children with recurrent episodes of protracted bacterial bronchitis (PBB), defined as a wet cough lasting at least 4 weeks which responds to antibiotics, are at risk for bronchiectasis, especially if they experience >3 episodes per year (38, 39). Lower airway infection with *H. influenzae* in patients with PBB was also found to be associated with

development of bronchiectasis (39). Also, a wet or productive cough which fails to respond to 4 weeks of oral antibiotics predicts the presence of bronchiectasis (38). Patients with these atypical cough patterns should undergo evaluation for bronchiectasis, including consideration of underlying etiologies including antibody deficiencies (**Table 1**).

Radiographically, high-resolution computed tomography (HRCT) is the most sensitive method for identifying structural lung abnormalities such as bronchiectasis. The predominant radiological feature of bronchiectasis is at least one dilated bronchus, defined as the internal luminal diameter of the airway exceeding the diameter of the adjacent vessel (9) (**Figure 3**). Additional key features include non-tapering of the bronchi as they traverse distally (**Figure 4**), and presence of visible bronchi within the outer 1–2 cm of the lung fields (9). While bronchiectasis is defined as a bronchoarterial ratio > 1, the normal anatomic ratio in children is much lower which may cause the diagnosis of bronchiectasis to be delayed or missed in this population (40). Some studies have suggested that the normal cut-off should be a ratio of 0.4–0.5 in infants and 0.8 in children less than age 18 years (9).

Among patients with antibody deficiencies, the decision of when to obtain the first HRCT and how often to repeat HRCT is a challenging exercise in clinical decision-making. In addition to the aforementioned clinical symptoms, other clinical characteristics and some immunologic abnormalities may be helpful in selecting which patients should receive a HRCT at the time their immunodeficiency is diagnosed. In one study, clinical factors which demonstrated a statistically significant correlation with the total CT score included length of respiratory symptoms before diagnosis of antibody deficiency, failure of immunoglobulin replacement therapy (IgRT) to establish adequate serum IgG levels, and FEV_1 and forced vital capacity (FVC) of <80% predicted (41). Other associated factors include older age or diagnostic delay (7, 32) and prior history of pneumonia (42). Immune evaluation has demonstrated a correlation of bronchiectasis in the setting of CVID with a CD4 count < 700 cells/microliter in peripheral blood (42). Other alterations in the lymphocyte subpopulations such as low IgM-memory B cells, class-switched-memory B cells, or total B cell number, also correlate with bronchiectasis (43). In patients with CVID, lower IgA (<7 mg/dL) (32) and lower IgM (mean 18 mg/dL with bronchiectasis vs. 26 mg/dL without bronchiectasis) (7) were associated with bronchiectasis (**Table 1**).

TABLE 1 | Factors associated with Bronchiectasis in patients with antibody deficiencies.

Delayed diagnosis of antibody deficiency
History of pneumonia
Prolonged respiratory infections
Chronic wet or productive cough
Protracted bacterial bronchitis, >3 episodes per year
Abnormal or worsening pulmonary function testing
Difficulty maintaining sufficient IgG trough on IgRT
CD4 count < 700 cells/microliter
Low B cells and/or Memory B cells
In the setting of CVID: very low IgA (<7 mg/dL) or very low IgM

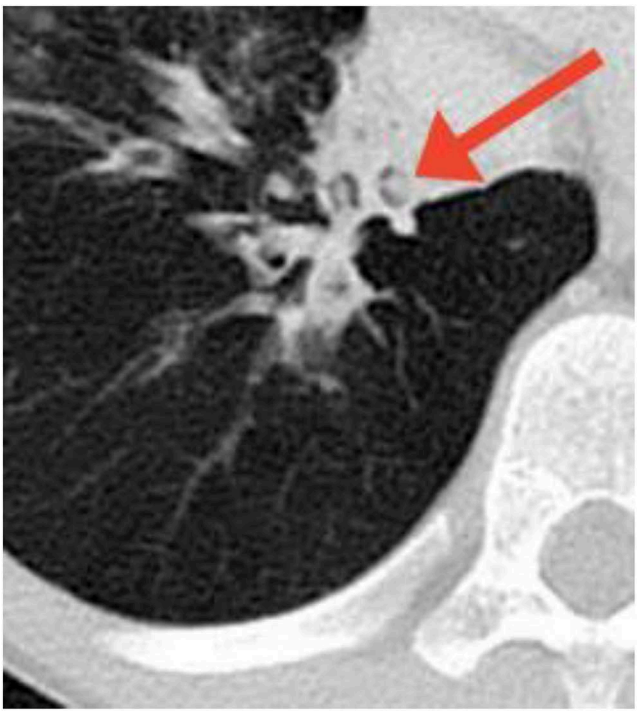

FIGURE 3 | Bronchiectasis in a child with a previously undiagnosed antibody deficiency. Multiple dilated bronchi are evident on CT. Arrow denotes the signet ring appearance of an ectactic bronchus containing a mucous plug and adjacent artery with an abnormal bronchoarterial ratio > 1. Image courtesy David A. Manning, MD, Radiologist, Children's Hospital New Orleans.

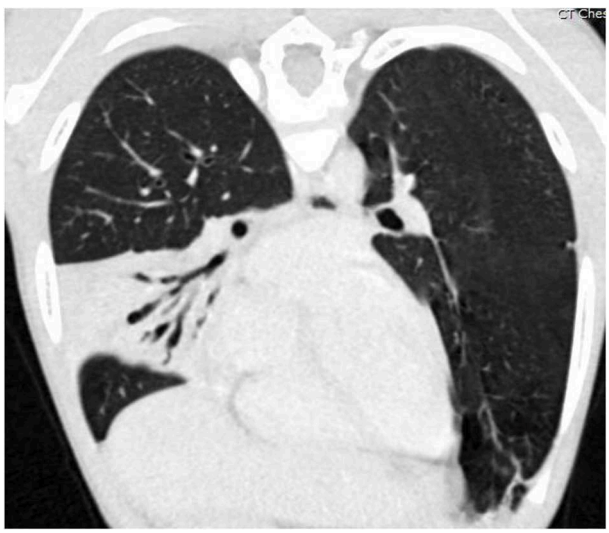

FIGURE 4 | Coronal oblique reformatted CT image of the chest of the same patient as **Figure 3**, demonstrating air bronchograms within a right middle lobe consolidation with varicose bronchiectasis. Image courtesy David A. Manning, MD, Radiologist, Children's Hospital New Orleans.

When deciding how often to repeat HRCTs, the clinician must consider that radiosensitivity and an increased risk for malignancy has been demonstrated in many forms of primary immunodeficiencies, including antibody deficiencies such as CVID (44–46). The cumulative dose of radiation due to diagnostic imaging that may be obtained in patients with a chronic condition could be excessive over time. A logical approach is to obtain HRCT no more often than 3–4 year intervals, unless an additional CT is needed to guide therapy or to evaluate new or worsening persistent symptoms (47). If a patient is maintaining a robust IgG trough level while on IgRT and is without chest infections, persistent cough, or decline in pulmonary function testing, it is appropriate to defer additional HRCT until a change in one of these factors is observed. Complete lung function testing including carbon monoxide diffusion at frequent intervals is a useful tool for monitoring the overall respiratory status in combination with clinical symptoms (47). Although pulmonary function testing is not sensitive enough to diagnose bronchiectasis, it can be useful for ongoing monitoring following the initial workup (47, 48). Any amount of ionizing radiation should be avoided unless it is absolutely essential to the care of the patient (49). It is important for the immunologist to lead the charge on this unique factor in the care of such patients, and to communicate the importance of radiosensitivity to the patient, pulmonologist, primary care provider, radiologist, and other team members.

To avoid exposure to ionizing radiation, magnetic resonance imaging (MRI) has been used among patients with exquisite sensitivity to radiation such as those with Ataxia-Telangiectasia, one of the many disorders involving DNA fragility or altered DNA repair mechanisms (37, 45, 49, 50). In addition to these patients, MRI has been shown to be comparable in detecting high and moderate grades of bronchial pathology among patients with CVID and may be considered as an alternative to HRCT (49, 51). Thoracic imaging with MRI has historically been underused due to technical barriers in obtaining high-quality images of the lungs but new techniques have been developed to overcome these challenges (52, 53). It is important to note that due to loss of signal in peripheral areas of the lung parenchyma, MRI is less sensitive than HRCT in detecting peripheral bronchial alterations (51). In addition, bronchiectasis may be more difficult to detect on MRI if not associated with a thickened wall or centered within an area of lung consolidation (53). While HRCT remains the most sensitive modality in detecting bronchiectasis (51, 54, 55), MRI can play an important role in monitoring patients with moderate or severe structural abnormalities (49, 52). In addition, MRI may be more sensitive in detecting early airway wall changes and mucus retention that may precede more serious structural changes such as bronchiectasis (56).

OPTIMIZING IMMUNOGLOBULIN REPLACEMENT THERAPY

It has been demonstrated that patients who continue to develop respiratory infections are at increased risk for progression of bronchiectasis (43). For patients who have an inability to make a strong antibody response, IgRT is the most important tool to protect against chest infections and subsequently slow this progression. Higher IgG trough levels, in addition to protecting against pneumonia (57), may also have a protective benefit against silent progression of bronchiectasis (31). Trough levels should never be allowed to fall below the physiologic normal for age. In fact, a trough of 1,000 mg/dL may be considered optimal for patients with bronchiectasis (31, 57). In children, a trough of 800 mg/dL may be sufficient, as long as they are not experiencing recurrent chest infections with their current IgRT plan. In addition to increasing the dose, techniques such as shortening the interval between infusions or changing to subcutaneous IgG (SCIg) replacement may be useful in achieving appropriate trough levels. Despite these recommendations for therapeutic trough levels, it is important to note that there is a wide range of IgRT doses which have been shown to keep patients with antibody deficiencies infection-free (58). In addition, patients with bronchiectasis may require twice as much replacement to achieve the same trough level as those without, possibly secondary to either increased losses or metabolism of IgG (58). A practical starting point would be no less than 0.6 g/kg/month and if the patient develops 3 or more infections a year, the dose should be increased (58). IgRT should thus be individualized and adjusted as necessary with a goal of preventing breakthrough infections rather than a sole focus on target trough levels (58). Despite treatment with immunoglobulin, patients with agammaglobulinemia and CVID may still develop chronic lung disease (59) and may need prolonged or prophylactic antibiotics in order to place a halt on the recurrent lung infections. It is essential for both the care team and the patient to understand that while IgRT is exceedingly important, an effective treatment plan for bronchiectasis is multifaceted. An aggressive multidisciplinary approach, discussed throughout this article, is also essential.

A MULTIFACETED APPROACH TO PULMONARY MANAGEMENT

General Principles

The central intent of the management of bronchiectasis is to minimize ongoing damage to the airways, and this is especially critical for pediatric patients, in whom the lungs are still developing (38, 60). With appropriately-intensive management and monitoring from subspecialist providers, and a commitment to self-care regimens from patients and families, the progression of bronchiectasis can be slowed, and the worst outcomes potentially avoided. It is important for each member of the multidisciplinary team to have at least a basic concept of the overall scope of the patient care plan. Clear communication with the patient and between team members is essential (**Figure 5**).

Bronchiectasis manifests over a spectrum of clinical severity, and pulmonary management is best accomplished when tailored to the individual patient. Current usual management may include the long-term use of inhaled pro-mucogenic and antibiotic therapies, inhaled bronchodilators, chronic anti-inflammatory use, physiotherapeutic airway clearance techniques, pulmonary rehabilitation services, and, in those cases where there is marked

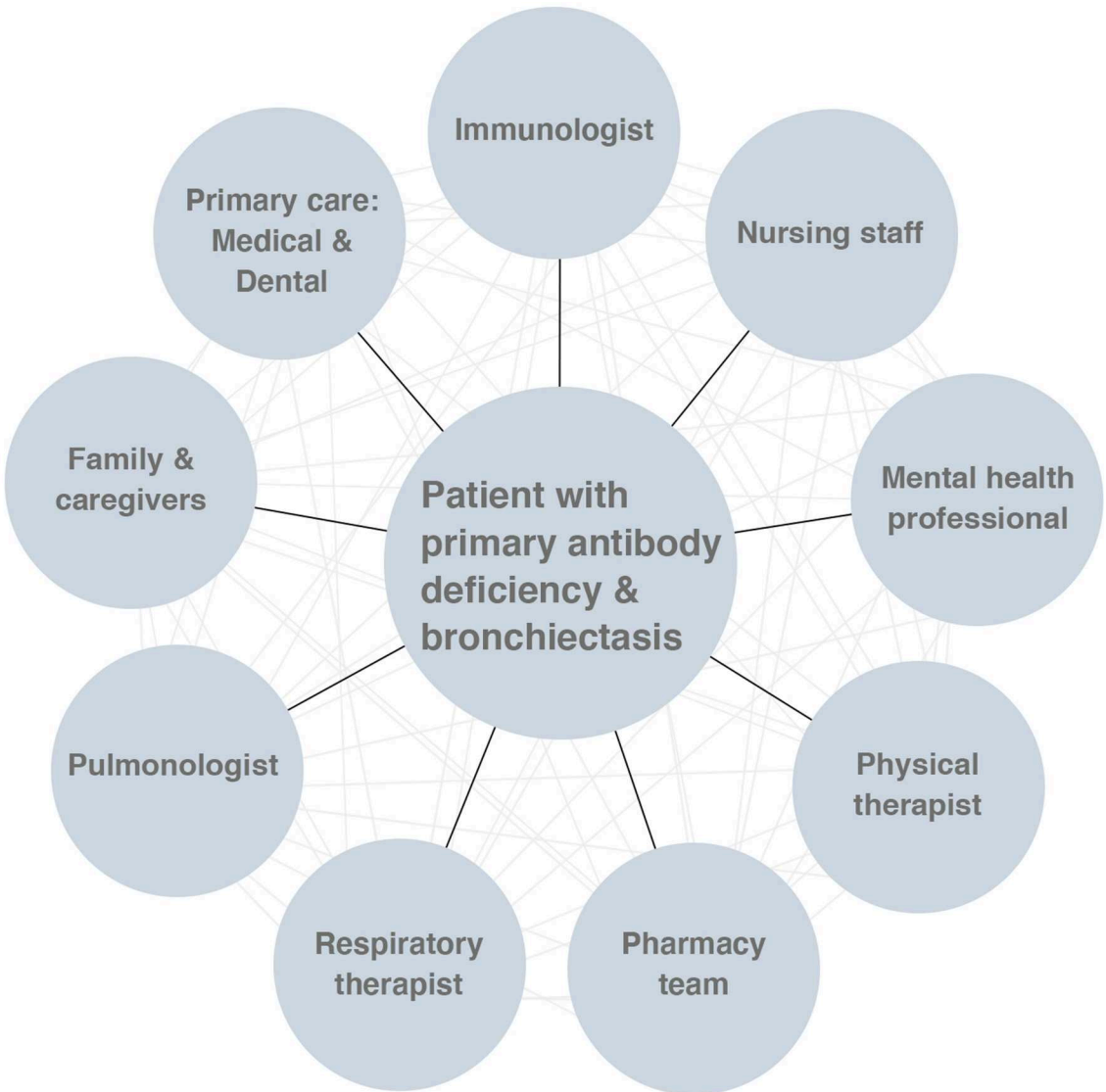

FIGURE 5 | The multidisciplinary approach to the care of patients with primary antibody deficiency and bronchiectasis.

structural damage, surgery (61). Optimal treatment consists of restoring or approximating normal airway clearance and facilitating mucociliary escalator function; and many patients will require acute interventions for intermittent bronchiectasis exacerbations. A typical approach to the use of multiple combined therapies is to use a short-acting bronchodilator to open the airways, followed by an inhaled muco-active hyperosmotic agent, then a mechanical airway clearance technique. Finally, after the airway has been optimized, inhaled medications (e.g., antibiotics) are used when appropriate (61). The best airway clearance regimen for the patient is not merely the one which is found clinically to be most effective, but also the one to which a given patient can best adhere. As such, the development of a patient's home regimen is a collaborative and longitudinal project between the patient and caregivers.

While the evidence base for the management of non-CF bronchiectasis is growing, there is a paucity of robust research regarding many of the techniques described below, particularly in the specific population of primary antibody deficiency (62). While many of these therapeutic approaches have well-established benefit in the CF population, this may or may not be portable to patients with non-CF bronchiectasis. A notable example of once such disjunction was the attempted use of recombinant DNAse in the non-CF bronchiectasis population, which was demonstrated in a clinical trial to actually increase the frequency of pulmonary exacerbations in these patients (63). To date, systematic reviews on the management of non-CF bronchiectasis have yielded only conditional recommendations based on relatively low-quality evidence. In light of the lack of strong randomized-controlled

trials of bronchiectasis therapies, much of the current standard of care for this disease is based on expert opinion and consensus. The European Respiratory Society (ERS) and British Thoracic Society have published recent guidelines on the management of bronchiectasis (61, 64). These important consensus statements form the basis for the subsequent discussion in this section. There are presently no consensus guidelines for the management of bronchiectasis in patients with primary antibody deficiency (62).

Mechanical Airway Clearance Therapies

The inspissated mucus of the bronchiectatic airway is a risk factor for bacterial colonization in the lung and contributes to the "vicious cycle" of infection and inflammation in patients (65). As mucociliary clearance is often dramatically reduced in non-CF bronchiectasis, several airway clearance techniques (ACTs), both provider- and self-administered, are deployed to help patients mobilize and expectorate mucus. While any of these techniques may be clinically and subjectively helpful to a given patient, it is important to note that most are not supported by robust evidence (66). Current expert consensus recommends that patients with chronic cough and difficulty expectorating be taught a preferred airway clearance technique, which they should perform twice daily as part of a broader airway clearance regimen (61, 67). There is currently no guidance as to which technique is preferred, and so a certain amount of pragmatism should be maintained: The best airway clearance technique is the one which works for the patient and to which they will adhere (67). Review of CT imaging may complement any manual physiotherapy regimen by focusing attention and time on the affected lobes (64).

High-Frequency Chest Wall Oscillation Therapy

High-frequency chest wall oscillation (HFCWO) therapy, performed either by a respiratory therapist with percussive panels connected to an air-pulse generator or by wearing a close-fitting inflatable vest similarly connected to an air-pulse generator, works by breaking up and thinning mucus in the airways through the oscillations of the chest wall and lung parenchyma. Though these devices have been studied extensively in the CF population, evidence is mixed and cannot be easily extrapolated to the non-CF bronchiectasis population (68).

Manual Chest Physiotherapy

Manual chest physiotherapy is a specialized airway clearance technique wherein a physical therapist or trained caregiver rapidly percusses the chest wall with cupped hands. This manual percussion and movement of the chest wall is usually combined with assisted postural drainage to optimize the proximal movement and subsequent expectoration of mucus. Systematic review and meta-analysis of this technique in CF patients demonstrated no advantage of manual chest percussion physiotherapy over other airway clearance techniques, including self-administered therapies (69). Nevertheless, when a patient is admitted to the inpatient service, a physical therapy consultation could be considered for assistance in airway clearance.

Expiratory Flow Modification and Autogenic Drainage

In addition to manual chest physiotherapy administered by a caregiver or therapist, patients may learn self-administered airway clearance techniques including specialized forced-expiration breathing patterns and self-directed postural drainage. These techniques are particularly helpful in that they are imminently portable (i.e., requiring no equipment) and the associated feelings of self-efficacy can be empowering to patients. These methods are comparable in clinical efficacy (including measures of pulmonary function and sputum production) to other airway clearance techniques, and may be preferred by patients (70).

Positive Expiratory Pressure Devices

Positive expiratory pressure devices work by providing slight resistance against exhalation, which further opens mucus-impacted airways through gentle additional positive end-expiratory pressure (PEEP). Some patients prefer to use handheld vibratory positive expiratory pressure devices, which similarly provide distending PEEP while adding the element of vibratory airflow which is thought to help break up mucus and move it up the mucociliary ladder. PEEP devices have compared favorably to HFCWO therapy in systematic review, particularly in terms of reduction in pulmonary exacerbation frequency and in patient satisfaction (71).

Bronchoscopy

Though not a part of routine bronchiectasis management, select patients may benefit from early bronchoscopy with lavage. When clinically warranted, flexible bronchoscopy with therapeutic lavage serves the dual role of permitting some mechanical clearance of mucus in the larger airways while generating potentially useful bronchial lavage samples for cellular and microbiologic analysis. Close communication with the attending anesthesiologist to promote lung recruitment during sedation and recovery is necessary.

Exercise and Pulmonary Rehabilitation

Maintenance of daily physical activity, optimally including some element of aerobic exercise, is an important part of bronchiectasis management. Current consensus recommendation is that adult bronchiectasis patients with impaired exercise capacity should undergo a pulmonary rehabilitation program in addition to daily airway clearance therapies (61). Indeed, this single recommendation from the 2017 ERS consensus statement was the only deemed to be supported by high-quality evidence (61, 64). When hospitalized, it may be helpful to consult physical therapy for exercise, if they are not already involved in the case for airway clearance.

Inhaled Therapies

Inhaled therapies, both as nebulized solutions and small-particle inhalers, are an attractive mode of medication delivery, as they offer deposition of medication directly to the airways. In the management of bronchiectasis, this is potentially complicated by the heterogeneity of ventilation and mucus in the airways;

nevertheless, inhaled medications are a central component to most management regimens.

The use of these medications, which are generally scheduled twice daily to co-occur with a mechanical airway clearance therapy, is time-consuming and difficult to perform outside of the home. As a consequence, inhaled medication regimens should be tailored to the individual patient, with consideration for the patient's lifestyle, preferences, and severity of disease. Current expert consensus suggests testing patient tolerance to a given inhaled agent before committing to a course of therapy (61). Broadly, the most common adverse effects of these medications are throat irritation and bronchoconstriction, and premedication with a beta-agonist is reasonable in select cases (61).

Hyperosmolar Agents and Mucolytics

The use of inhaled mucolytic medications and hyperosmolar solutions is central to bronchiectasis care in the management of CF, however the current evidence base is mixed in non-CF bronchiectasis (72). Nevertheless, expert consensus favors the use of nebulized pro-mucogenic agents in those non-CF bronchiectasis patients with difficulty adequately mobilizing secretions (61, 64). Of note, by design, these mediations aid with the mobilization and expectoration of mucus, and occasionally patients perceive the resultant increase in mucus production as aversive.

Nebulized saline, generally as a hypertonic solution (>0.9% w/v, and usually as 3 or 7% in clinical practice), hydrates airway mucus by osmotic action, pulling water from the airway epithelium, improving mucus viscosity, and enhancing mucociliary clearance. Patients generally perform nebulizer treatments twice daily concurrent with their preferred airway clearance technique, with each treatment taking up to 30 min. One randomized single blind cross-over study of 28 adult patients with non-CF bronchiectasis found a clinically meaningful and statistically significant improvement in FEV_1, FVC, and a QOL score, and reduced antibiotic usage and emergency health care utilization (73). In patients with milder disease, isotonic saline, or even sterile water may be equally effective and may be better tolerated in those patients who experience bronchospasm with hypertonic saline (64, 72).

Recombinant DNAse (dornase alfa), works principally by lysing extracellular DNA aggregates resulting from the NETosis of neutrophils, which makes it attractive for use in disorders which involve a dysregulated neutrophil response, such as bronchiectasis. Unfortunately, while DNAse is an impactful therapy for many CF patients, it was associated with an increased frequency of exacerbations and worsened FEV_1 in a clinical trial in idiopathic bronchiectasis patients (63). As such, its use in the adult non-CF bronchiectasis population is *explicitly discouraged* by current guidelines (61, 64).

Inhaled Bronchodilators

The use of short-acting inhaled bronchodilators prior to a patient's airway clearance regimen is reasonable, as this both opens the airways prior to subsequent inhaled therapies and mechanical airway clearance and may limit bronchoconstriction related to the use of inhaled hyperosmotic agents and antibiotics. The use of long-acting bronchodilators as a maintenance therapy should not be routinely offered to patients without another strong clinical indication (e.g., significant breathlessness, COPD) (61).

Inhaled Antibiotics

A national registry of non-CF bronchiectasis patients in the United States of America found that *P. aeruginosa, Staphylococcus aureus, H. influenzae,* and non-tuberculous *mycobacteria* (NTM) are the commonest bacterial pathogens in this population (74). Long-term courses of inhaled antibiotics (e.g., colistin, tobramycin, and gentamycin) should be considered for patients with *P. aeruginosa* infection and 3 or more pulmonary exacerbations in a year, or for those patients without *P. aeruginosa* who have frequent exacerbations which are macrolide-refractory, or for those patients who are intolerant of oral macrolides (61). There is no strong evidence favoring oral vs. inhaled antibiotics in bronchiectasis, and so clinical judgement must be used in concert with attention to patient preferences (75). Possible adverse effects from inhaled antibiotics used in bronchiectasis include throat irritation and dysgeusia, chest discomfort and bronchoconstriction, and cough. Serum levels are generally not required for monitoring of therapeutic index when using inhaled antibiotics (i.e., aminoglycosides) alone in patients without underlying renal disorders, but should be considered if there is clinical evidence of nephrotoxicity or ototoxicity (e.g., tinnitus) (76, 77).

Inhaled Corticosteroids

As inflammation is central to the pathomechanism of bronchiectasis, it seems reasonable to include an inhaled corticosteroid (ICS) in the management of these patients. To date, relatively small trials of ICSs in bronchiectasis patients have not demonstrated benefit in either reducing the frequency of exacerbations or in improving pulmonary function or QOL scores. Nevertheless, ICS use is rather common in adult patients with bronchiectasis (78). A Cochrane review of seven studies involving 380 adult patients concluded that there was insufficient evidence to support use of ICS in adult bronchiectasis patients (79). This conclusion is consistent with current consensus expert guidelines, which state that ICS should not be routinely offered to adults with bronchiectasis, but that patients with comorbid asthma or chronic obstructive pulmonary disease (COPD) should continue these medications if deemed clinically useful (61, 64).

Systemic Therapies

Systemic Antibiotics for Acute Exacerbations

Systemic antibiotics, generally taken orally, are a mainstay in the management of acute exacerbations of bronchiectasis. Optimally, the antibiotic management of bronchiectasis is informed by the patient's own usual pulmonary pathogens and chronic colonizers. When clinically feasible, a sputum sample should be obtained prior to initiation of antibiotic therapy to surveil for changes in susceptibility patterns. Additionally, a functional understanding of any underlying immune defect may inform antibiotic choice and duration of therapy. However, in clinical practice empiric antibiosis is often pursued. In this case,

macrolides (e.g., azithromycin, erythromycin) are considered first-line and have been shown to significantly reduce the frequency of pulmonary exacerbations (61, 80). Current expert consensus recommends 14-day courses of systemic antibiotics for bronchiectasis exacerbations in adults, citing no direct evidence of benefit from longer courses, and the need to balance patient preference and the risk of inducing antibiotic tolerance (61). Systematic review of controlled trials examining the utility of concurrent use of inhaled and oral antibiotics for exacerbations found no evidence of benefit for this wide-spread practice (81).

While induced sputum samples may yield helpful cultures in older patients, in younger patients sputum cultures may over-represent oropharyngeal flora. Flexible bronchoscopy with thorough lavage can yield directive bacterial culture and antibiotic susceptibility panels, though it should be remembered that there is potentially marked heterogeneity in bacterial sub-populations within different segments of the airways and lungs.

Eradication antibiotic protocols for those patients with newly-identified *P. aeruginosa* should be pursued (61). This recommendation is due to the markedly deleterious effect of *P. aeruginosa* on the rapidity of decline in pulmonary function in bronchiectasis patients. *P. aeruginosa* eradication regimens generally involve a two-week initial treatment phase with oral or intravenous antibiotics and an adjunctive inhaled antibiotic (e.g., colistin, tobramycin, or gentamicin), followed by an additional 10 weeks of inhaled antibiotics. Such a regimen is not currently recommended for other pathogens associated with bronchiectasis.

Systemic Antibiotics for Prophylaxis

As discussed previously, despite appropriate immunoglobulin replacement, many patients with antibody deficiencies will still develop bronchiectasis. Patients who should be considered for prophylactic antibiotics include those with three pulmonary infectious exacerbations in 1 year (61, 82, 83) or who have declining lung function despite appropriate immunoglobulin replacement (84, 85). Because macrolide antibiotics have anti-inflammatory and anti-microbial properties, they are most commonly used for prophylaxis in patients with bronchiectasis (86–88). Typical dosing includes 5 mg/kg for children or 250 mg/day for adults three times per week (2, 89). The use of prophylactic antibiotics in patients with primary immunodeficiency and bronchiectasis is primarily extrapolated from CF and non-CF bronchiectasis, as there is little published data specifically on patients with antibody deficiencies. A Cochrane review in 2015 evaluated the evidence behind courses of prolonged ($\geq$1 month) antibiotic therapy for non-CF bronchiectasis (90). This meta-analysis of 18 randomized controlled trials and 1,157 patients (including mainly adults and a smaller subset of children) suggested that prolonged antibiotic use was associated with a significant reduction in the number of reported bronchiectasis exacerbations, and a non-statistically significant reduction in hospitalization. Overall, the authors found that there is evidence of moderate quality to suggest that the use of prolonged antibiotics may benefit patients with non-CF bronchiectasis, however they did raise concerns about the potential for emergence of antibiotic tolerance with this approach (90). Subsequent systematic reviews and meta-analyses reaffirmed prior findings of a reduction in exacerbation and improvement in QOL scores and pulmonary function indices (82, 91). Two large recent randomized control trials, the EMBRACE (Azithromycin for prevention of exacerbations in non-CF bronchiectasis) and BAT (Bronchiectasis and Longterm Azithromycin Treatment) trials, also endorsed the role of azithromycin in non-CF bronchiectasis, the latter of which included five patients with CVID (92, 93). Due to the risks of antimicrobial resistance, treatment should be discontinued after a trial of 3–6 months if there is no clear evidence of benefit (83). Prior to initiating long-term macrolides, it is important to rule out non-tuberculous mycobacterium (64).

Immune-Modulating and Anti-inflammatory Medications

Vaccination is an important consideration in the preventative care of children and adults with bronchiectasis. Expert opinion recommends giving the seasonal *influenza* vaccination, as well as ensuring maintenance of vaccination against *S. pneumonia, H. influenzae*, and *Bordetella pertussis* when appropriate for the patient and not otherwise contraindicated (94–97). Most patients with antibody deficiencies are maintained on IgRT and are receiving antibody protection passively. Therefore, the above-mentioned vaccines are not indicated while on an IgRT plan, apart from influenza vaccination. Because influenza virus antigens change frequently and there is a significant delay between the time of donor IgG collection to the production of commercially available IgRT, IgRT might not contain the most seasonally relevant anti-influenza antibodies (98).

Surgical Management

In some patients, bronchiectasis may develop in sub-segmental airways to an extent where distal lung segments are no longer functional. These areas may then act as a reservoir for chronic infection, the development of highly resistant pathogens, and episodic reinfection of adjacent lung units. When such disease is highly localized, and when optimal medical management has failed to improve a patient's pulmonary health, surgical resection of the affected lobe (or, less commonly, segmentectomy or pneumonectomy) can be considered. Advances in the medical management of bronchiectasis have made surgical intervention less common. However, in the case of highly-localized disease, resection of affected lobes can improve a patient's QOL and reduce the frequency of antibiotic courses (99, 100). Indication for surgical resection of an affected lobe of the lung include persistent failure of medical management and hemoptysis, and current expert consensus recommends limiting lobectomy to those patients in whom maximal medical management has been trialed (61, 100).

Pre-operative management should include 1–2 weeks of targeted antibiosis to avoid complications including bronchopleural fistula and empyema (101). Generally, performance of contrast-enhanced CT to reassess the extent of disease and aid surgical planning is warranted (101). It is important to note that, following any surgical intervention to the upper airway, chest, or abdomen, pain may limit effective airway

clearance through cough, and the use of opioid pain medications could suppress the respiratory drive, particularly during sleep. In these cases, a physical therapy consultation can assist with adaptive airway clearance techniques (e.g., cough splint) during recovery to minimize atelectasis.

One consideration for those patients in whom aspiration is felt to be a driver or major contributor to the treatment-refractoriness of their bronchiectasis, is correction of anatomical issues promoting reflux and aspiration, including fundoplication and laryngeal cleft repair (102–104). Aspiration may cause both direct tissue damage and bacterial inoculation of the airways, but could also modulate the growth and lifestyle patterns of pathogens in the airways, driving them to more virulent or chronic expression patterns (105). Prokinetic medications and conservative anti-reflux precautions should be considered first (64).

Lung transplantation referral may be considered for those patients 65 years old or less with $FEV_1 < 30\%$ predicted, or in those patients with rapid functional decline and severe pulmonary hypertension (pHTN), massive hemoptysis, or respiratory failure (64). Lung transplantation has been used in patients with CVID with variable results (28, 47, 106).

Management of Extra-Pulmonary Complications of Bronchiectasis

Patients with bronchiectasis may have significant comorbid extra-pulmonary health issues, including failure to thrive and psychosocial problems. Primary antibody deficiency patients with bronchiectasis are at increased risk for pHTN, and so echocardiography should be considered in those patients with dyspnea, exercise intolerance, and other signs of pHTN (107). Sinus disease is common in this population and should be managed when found as the sinus can act as a reservoir for sinopulmonary pathogens. Likewise, good dental hygiene and routine dental visits are important to reduce oral infections.

Longitudinal Follow-Up and Patient Adherence to Chronic Therapy

Patients with bronchiectasis generally warrant close follow-up, as daily airway clearance regimens are, by their nature, time-consuming, and often difficult to maintain. Clinic visits should include an honest, direct reappraisal of how the patient and family is *actually* performing their regimen, and the clinician should be prepared to troubleshoot when things are not working well or are impracticable. Close follow-up in clinic also permits the monitoring of pulmonary function, and a decline in FEV_1 and related indices may herald an impending exacerbation and provide an opportunity for early, outpatient intervention. While there is no specific consensus guidance for primary antibody deficiency patients with bronchiectasis, other subspecialty organizations recommend clinic visits as frequently as four times annually, and this may be appropriate for primary antibody deficiency patients with significant bronchiectasis (108). These visits should include repeat pulmonary function testing, sputum microbiology, and consideration of chest x-ray (64).

In one study of a population of 75 patients with non-CF bronchiectasis with a sputum culture positive for *P. aeruginosa*, researchers found that beliefs about the medical necessity and potential risks of medications and airway clearance techniques predicted adherence to therapy (109). Older age was associated with increased adherence, as was fewer medications in the regimen. A structured literature review published by the Cochrane group in 2015 found no studies which tested interventions to improve adherence, and we were not able to identify any subsequent clinical studies on this topic (110).

FUTURE DIRECTIONS IN BRONCHIECTASIS MANAGEMENT

Though much of the existing evidential support for therapies in non-CF bronchiectasis is relatively weak, the field of research has grown appreciably in the past few years, and several promising avenues of treatments have been identified. A recent review by Chalmers and Chotirmall has capably mapped the current state of the field by identifying 11 clinical trials comprising the cutting-edge of current research (111). Among these, perhaps the most mechanistically compelling are those targeting immune dysregulation and off-target effects in the bronchiectatic airway, such as the current trial of a human NE inhibitor (BAY 85-8501) (25). Doubtless, the evidentiary basis for our interventions will continue to strengthen in the coming years as these clinical trials progress. New patient registries will be invaluable in drawing new insights into this previously under-studied disease (112).

To best guide management and anticipate bronchiectasis exacerbations, new diagnostic and monitoring techniques will need to be developed. The addition of molecular techniques to sputum microbiology assays offers improved sensitivity in pathogen detection, and a window into the complex pulmonary microbiome (113). One study of sputum NE activity in a large population of adults with bronchiectasis found an association of increased activity and future risk of exacerbation, offering the promise of an easily-obtainable bio-marker for clinical surveillance (24, 114). There is robust research presently underway to identify clinical phenotypes and so-called "treatable traits" in non-CF bronchiectasis, which will one day permit more precise and tailored management strategies (115, 116).

As it is likely that daily maintenance interventions for bronchiectasis will remain relatively involved for patients and caregivers despite these ongoing advancements, it will be important to study ways to foster adherence in this population (110). Chronic bronchiectasis and primary antibody deficiency are burdensome diseases for patients and their families, and research into psychosocial interventions for these patients is needed. Expansion of multi-disciplinary care for patients with primary antibody deficiency-associated bronchiectasis (i.e., building teams of immunologists, pulmonologists, nurses, mental health professionals, case-workers, and respiratory and physical therapists), is critical to ensuring that health outcomes for these patients meet or exceed those for patients with bronchiectasis secondary to other etiologies (117–119) (**Figure 5**).

AUTHOR CONTRIBUTIONS

LW designed the concept of the manuscript, contributed to the writing, and editing of the manuscript. EW contributed to the writing and editing of the manuscript. KG contributed to the writing and editing of the manuscript, and the design of figures. RS provided oversight and critical review of the manuscript.

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Serum Free Light Chains in Common Variable Immunodeficiency Disorders: Role in Differential Diagnosis and Association With Clinical Phenotype

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We report on an observational, multicenter study of 345 adult CVID patients, designed to assess the diagnostic value and the clinical association of serum free light chain (sFLC) pattern in Common Variable Immunodeficiency disorders (CVID). Sixty CVID patients were tested twice in order to assess intraindividual variability of sFLC. As control groups we included 138 patients affected by undefined primary antibody defects (UAD), lymphoproliferative diseases (LPDs), and secondary antibody deficiencies not related to hematological malignancies (SID). CVID patients presented lower κ and λ chain concentration compared to controls, showing low intraindividual sFLC variability. On the basis of the sFLC pattern, patients were classified into four groups: κ−λ+, κ+λ−, κ−λ−, κ+λ+. The most common pattern in CVID patients was κ−λ− (51%), followed by κ−λ+, (25%), κ+λ+ (22%), and κ+λ− (3%). In UAD, LPD, and SID groups κ+λ+ was the most common pattern observed. By analyzing the possible association between sFLC patterns and disease-related complications of CVID, we observed that patients belonging to the κ−λ− group presented more commonly unexplained enteropathy compared to the κ+λ+ group and showed higher frequency of bronchiectasis and splenomegaly compared to both the κ−λ+ and κ+λ+ patients. When compared to the other groups, κ−λ− had also lower serum IgG, IgA, and IgM concentrations at diagnosis, lower frequency of CD27+IgD–IgM– switched memory B cells, and higher frequency of $CD21^{low}$ B cells, receiving earlier CVID diagnosis. Thus, lower levels of sFLC might be an epiphenomenon of impairment in B cell differentiation, possibly leading κ−λ− patients to a higher risk for bacterial infections and chronic lung damage. Based on these results,

we suggest adding sFLC assay to the diagnostic work-up of hypogammaglobulinemia and during follow-up. The assay may be useful to differentiate CVID from other causes of hypogammaglobulinemia and to early detect monoclonal lymphoproliferation occurring over years. Moreover, since the sFLC pattern seems to be related to disease phenotypes and clinical manifestations of CVID and after confirmation by further studies, sFLC assay might be considered a promising prognostic tool for identifying patients at higher risk of developing enteropathy and chronic lung damage or splenomegaly. This will allow designing a tailored follow-up for CVID patients.

Keywords: serum free light chains, common variable immunodeficiency disorders (CVID), lymphoproliferative disease (LPD), diagnostic marker, immunoglobulin, clinical phenotype, non-infectious complications

BACKGROUND

Common variable immunodeficiency disorder (CVID) is the most common symptomatic primary antibody deficiency (PAD) diagnosed in adulthood, affecting 1:25,000–1:50,000 patients in western countries (1, 2). Due to the lack of confirmative test, diagnostic criteria include low circulating immunoglobulin count (decrease in IgG and IgA, +/– IgM), exclusion of other causes of hypogammaglobulinemia, and a list of other immunologic parameters (e.g., poor response to vaccination, low percentage of switched memory B cells) that can differentiate CVID from unclassified antibody deficiency (UAD) (https://esid.org/Education/Diagnostic-Criteria-PID). From a clinical point of view, manifestations of CVID are extremely heterogenous, leading to different clinical phenotypes and prognosis, with patients without complications showing better survival (3, 4).

Immunoglobulins are formed by a double pair of identical heavy and light chains, these latter being kappa (κ) or lambda (λ) chains. Free light chains are usually detectable in the serum (sFLC) due to an excess in production (around 40%) compared to heavy chains (5). A serum FLC (sFLC) nephelometric assay has been developed almost 20 years ago to accurately measure the amounts of circulating serum free κ and λ light chains (6). Serum and/or urine testing for monoclonal free light chains, together with the presence of an M-protein (paraprotein), is critical to the diagnosis of multiple myeloma (MM). In rare cases, malignant plasma cells, such as in MM and related disorders, secrete only a clonal light chain without any identifiable heavy chain. Monoclonal increase of κ or λ chain serum levels, leading to an abnormal serum FLC ratio (FLCr), may thus reveal the presence of a non-secretory myeloma in case a paraprotein is absent, thus reducing diagnostic delay (7). In the case of immunoglobulin light chain (AL) amyloid deposition, sFLC assay has been shown to be more sensitive for diagnostic purposes than conventional serum electrophoresis or immunofixation (5). Besides plasma cells dyscrasias, a significant imbalance in sFLC secretion has been suggested as a sensitive indicator of B-cell clonality and as a prognostic marker in chronic lymphocytic leukemia (B-CLL), monoclonal B-cell lymphocytosis (MBL), and some B-cell non-Hodgkin lymphomas (NHL) (8–10). Apart from revealing the presence of a clonal, κ- or λ-restricted cell population, sFLC absolute values correlate with disease activity and clinical outcome in patients with plasma cell malignancies (11). Moreover, increased sFLC levels with a normal FLCr have been suggested as a useful marker of the polyclonal B-cell activation and/or expansion sustaining the activity of different chronic inflammatory and autoimmune conditions (12). Finally, an impaired sFLC catabolism may be an indirect signature of a progressive renal injury (13).

Being a sensitive, simple, and reproducible indicator of B-cell biologic activity, the potentiality of sFLC assay has been more recently explored in the context of primary B-cell impairments/deficiency, including PAD and particularly CVID.

In 2012, Unsworth et al. first reported in a small cohort of PAD patients extremely low levels of κ and/or λ chains (below the limits of reliable detection) in 19/20 cases, mostly CVID, leading to impaired or non-calculable serum FLCr. Thus, they suggested that a suspicious κ/λ ratio, usually due to very low absolute quantities of at least one sFLC, most likely underlies the disease-related B-cell dysfunction, rather than a B lymphocyte clonality in this specific setting (14).

We subsequently reported a possible role of sFLC in the differential diagnosis between a primary and secondary hypogammaglobulinemia, suggesting their possible role in risk stratification of adults with CVID, in terms of peculiar biological characteristics, clinical behavior, and prognosis (15).

Recently, sFLC have been described as a marker for differentiation of PAD, with a high specificity but limited sensitivity for CVID. A possible prognostic and therapeutic relevance has also been hypothesized, reporting significant results in a single center cohort of 81 CVID patients (16).

Finally, rare cases of primary immunoglobulin κ light chain defects have been reported since 1972, whose genetic basis has been extensively explored only in two cases (17). κ chain deficiency is included in the 2017 IUIS Phenotypic Classification for Primary Immunodeficiencies between PADs, reported as asymptomatic (18).

Herein we report the results of an observational multicenter study of sFLC levels in a cohort of 345 CVID patients. sFLC levels have been correlated with clinical and laboratory features of CVID. sFLC pattern of CVID patients has been compared to that of three different cohorts of non-CVID patients referred due to a first recognition of hypogammaglobulinemia, further characterized as secondary to lymphoproliferative diseases

(LPDs), secondary to protein loss or medications (SID) or PAD other than CVID (unclassified antibody defects, UAD). Our findings suggest a diagnostic and potential prognostic role of sFLC assay that might be taken into account when designing personalized follow-up strategies and support its inclusion in the diagnostic work-up of CVID and other forms of hypogammaglobulinemia.

METHODS

Study Population

We enrolled 345 adult patients (>18 y.o.) with a diagnosis of CVID (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). All patients were regularly followed in inpatient and daycare settings by University Hospitals working as referral centers for adult primary immune deficiencies in Rome, Naples, Padua, Udine, Florence, and Bari and included in the IPINET Italian Registry for CVID. Exclusion criteria included inability or unwillingness to provide written informed consent. As control groups, we also included in the analysis 59 unclassified antibody defects (UAD) and 79 patients initially referred to the Padua Centre due to hypogammaglobulinemia, subsequently diagnosed as lymphoproliferative diseases (LPDs, $n = 41$) or as secondary antibody deficiencies unrelated to hematological malignancies (SID, $n = 38$). For UAD definition we used the criteria provided by the ESID registry, including patients with clinical features of PAD, marked decrease of at least one Ig isotype/IgG subclass or failure of IgG response to vaccines, who did not fit any other working definition (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). All patients enrolled provided their informed consent. All the patients of the control groups underwent sFLC analysis for diagnostic purposes at the Department of Laboratory Medicine of the University Hospital of Padova. The Ethical Board of Padua approved this study, that was performed in accordance with the Good Clinical Practice guidelines, the International Conference on Harmonization guidelines, and the most recent version of the Declaration of Helsinki (15).

Study Design

Observational, multicenter study to assess the diagnostic and prognostic value of FLC in CVID. Once the informed consent form was signed, the investigator reported participant's demographic, clinical, and laboratory data in the case report form. A set of variables was recorded for each patient including gender, date of birth, date of detection of hypogammaglobulinemia, immunoglobulin serum levels at diagnosis. Only for CVID patients we collected clinical phenotype according to the Chapel et al. proposal (19). CVID-associated conditions (cytopenia, unexplained persistent proliferation, unexplained persistent enteropathy, bronchiectasis, splenomegaly, cancer, autoimmunity, GLILD) and B-cell phenotype performed according to Wehr et al. were also registered (20). At the time of the enrollment, the serum sample for FLC assay collected during routine blood tests was then sent to the Department of Laboratory Medicine of the University Hospital of Padova as referral center (21). A second determination of sFLC concentration was available for 60 patients, with samples collected 24 ± weeks after the first time, and results were analyzed in order to assess the intraindividual variability of sFLC assay. Only patients with at least one available measurement of sFLC were finally included in the database for data analysis. CVID patients were all on immunoglobulin replacement treatment (IgRT). We included patients independently on IgRT since it has been previously demonstrated that sFLC concentration is not affected by Ig replacement (15, 16).

sFLC Assessment

According to standard diagnostic procedure, serum samples were stored at −80°C until FLC assay was performed. FLC (Freelite^{TM} κ and λ, Binding Site, UK) assay was applied on a BNII Nephelometer (Siemens) (22). Previously established reference ranges were used [4.52–22.33 and 4.84–21.88 mg/L for κ FLC and λ FLC, respectively and 0.44–2.67 for FLC ratio (FLCr)] (6, 21). On the basis of sFLC concentration, patients with CVID were classified into four groups: in three groups κ (κ−λ+ pattern), λ (κ+λ− pattern), or both (κ−λ− pattern) light chains were reduced or undetectable; in the fourth group they were normal (κ+λ+). Patients with more than one (previous or subsequent) available sFLC measurement did not show any significant change in pattern over time, as previously reported, unless in concomitance with a new onset of hematological malignancy (15).

Statistical Analysis

Patient demographics and clinical characteristics were summarized by frequencies and percentages, with means and standard deviations or median and 5–95th centile where appropriate. Comparisons of continuous parameters between treatment groups were calculated with a *t*-test if normally distributed and with a Mann–Whitney *U*-test if not normally distributed. Normal distribution of the data was evaluated by Kolmogorov–Smirnov test. Differences in frequencies between groups were calculated using the Fisher's exact test. Comparison between continuous parameter and sFLC concentration was assessed by simple linear regression analysis. Intraclass correlation coefficient (ICC) was used to evaluate intraindividual variability of both κ and λ chain serum concentration between the two available assessments. ICC values between 0.4 and 0.6, 0.6, and 0.8 or ≥0.8 were considered to indicate moderate, good, or very good agreement, respectively (23). Data were analyzed by using group sequential testing that allowed "spending" a little of the α value at each interim analysis such that the total type I error did not exceed 0.05 at the end of the study. Statistical analyses were performed with the statistical package SPSS (IBM SPSS Statistics for Windows, version 25.0; IBM, Armonk, NY).

RESULTS

Baseline Characteristics

sFLC concentrations were measured in 345 CVID patients and in 138 controls (59 UAD, 41 LPDs, and 38 SID subjects). The

TABLE 1 | Demographics and immunoglobulin serum levels at diagnosis in the cohort included in the analysis.

	CVID ($n = 345$)		UAD ($n = 59$)		LPDs ($n = 41$)		SID ($n = 38$)	
Age, years, mean (SD)	49.4	(14.3)	53.6	(16.8)*	67.2	(11.2)****	56.1	(14.9)***
Sex (female), *n* (%)	169	(49)	36	(62)	48	(40)	17	(55)
Immunoglobulin serum level at diagnosis of hypogammaglobulinemia								
IgG, g/L, median (IQR)	3.1	(1,9–3.4)	5.9	(5.2–6.7)****	5,3	(4.2–8.2)****	5.9	(5.2–6.7)****
IgA, g/L, median (IQR)	0.1	(0.1–0.3)	1.2	(0.9–1.6)****	0.4	(0.2–1.3)****	1.3	(1.0–1.8)****
IgM, g/L, median (IQR)	0.2	(0.1–0.4)	0.9	(0.4–1.1)****	0.3	(0.4–1.1)	0.5	(0.3–1.0)****

*Levels of significance for comparison with CVID are: *P ≤ 0.05, ***P ≤ 0.001, ****P ≤ 0.0001. Continuous variables were analyzed by t-test if normally distributed (age) or by u-test (Ig serum levels); sex was analyzed by Fisher's test.*

TABLE 2 | Clinical characteristics of 345 CVID patients enrolled.

Chapel phenotype, n (%)		
Infection only	177	(52)
Cytopenia	68	(20)
Lymphoproliferation	113	(33)
Enteropathy	48	(14)
CVID-related complications, n (%)		
Splenomegaly	151	(44)
Bronchiectasis	105	(31)
Autoimmunity	114	(33)
ITP/AHE	38	(13)
Psoriasis	14	(4)
Vitiligo	15	(4)
Celiac disease	10	(3)
Other	44	(13)
GLILD	28	(8)
Malignancies	53	(15)
Lymphomas	16	(5)
Gastric cancer	13	(4)
Breast cancer	4	(1)
Colorectal cancer	4	(1)
Other	17	(5)

characteristics of the cohorts are recapitulated in **Table 1**. The mean age of CVID at study enrollment was 49.4 ± 14.3 years, lower than what was recorded in UAD (53.6 ± 16.8 years, *t*-test, $p = 0.050$), LPDs (67.2 ± 11.2 years, *t*-test, $p < 0.0001$), and SID (56.1 ± 14.9, *t*-test, $p = 0.016$). As expected, CVID patients also displayed lower immunoglobulin serum levels at diagnosis of hypogammaglobulinemia in comparison to the other groups (**Table 1**). Diagnosis for patients classified as LPDs and SID are detailed in **Table S1**.

As shown in **Table 2**, 52% of CVID patients displayed an infection-only phenotype, as defined by Chapel et al. (19), whereas at least one CVID-related complication was found in the remaining subjects, namely cytopenia (20%), unexplained lymphoproliferation (33%), unexplained enteropathy (14%). Thirty-three percent of participants have had at least one autoimmune complication and 15% had a past medical history of cancer.

Serum Free Light Chains and IgA and IgM Serum Levels

In CVID, the median sFLC value was 2.0 mg/L (IQR 0.3–9.1) for κ chains, and 6.2 mg/dl (IQR 0.0–10.2) for λ chains, and the FLCr was 0.9 (IQR 0.6–1.4). As shown in **Figure 1**, CVID patients displayed lower κ and λ chain serum levels in comparison to UAD, SID, and LPDs (*u*-test $p < 0.0001$ for all comparisons). Sixty CVID patients tested twice for sFLC concentration presented a very good intraindividual agreement between the first and the second determination of both κ and λ chains (ICC 0.9 and 0.8, respectively).

CVID group showed lower serum FLCr in comparison to LPDs (*u*-test, $p = 0.039$, **Figure 1C**). However, 142 (41%) of CVID participants displayed an unbalanced FLCr. Among those, one patient showed very high concentration of κ chains (811 mg/L) with a FLCr of 62.9, and he was diagnosed with NHL few weeks after the study enrollment; a second patient presented a similar history. In all other patients, the very low/undetectable concentration of at least one sFLC was the reason for κ and λ chain imbalance, in agreement with previous findings (14). On the contrary, 82% of patients in the LPDs group had unbalanced FLCr due to an underlying MM or B-CLL. In addition, we observed low frequency of unbalanced serum FLCr in the SID and UAD groups, respectively 3 and 12% of subjects.

When analyzing immunoglobulin serum levels at diagnosis in the different cohorts, IgA levels were directly associated with serum κ and λ chain concentrations in CVID (R^2 0.03 $p < 0.0001$ and R^2 0.07 $p < 0.0001$, respectively) and UAD (R^2 0.25, $p < 0.0001$, R^2 0.25, $p < 0.0007$), but not in LPDs and SID (**Figures S1A,B**). Only in the CVID cohort IgM was directly associated with both serum κ and λ chain concentrations (R^2 0.46 $p < 0.0001$ and R^2 0.05 $p < 0.0001$, respectively—**Figures S2A,B**). No association was found between sFLC serum concentration and IgG in CVID and in control groups.

sFLC Pattern

Based on sFLC reference values, patients were grouped into four phenotypes (**Figure 2**). For CVID, the most common pattern was k–λ– (51%), followed by k–λ+ (25%), k+λ+ (22%), and k+λ– (3%). Differently from CVID, in the UAD, LPD, and SID groups, the κ+λ+ was the most common pattern observed, including, respectively 83 ($p < 0.0001$), 70 ($p < 0.0001$), and 90%

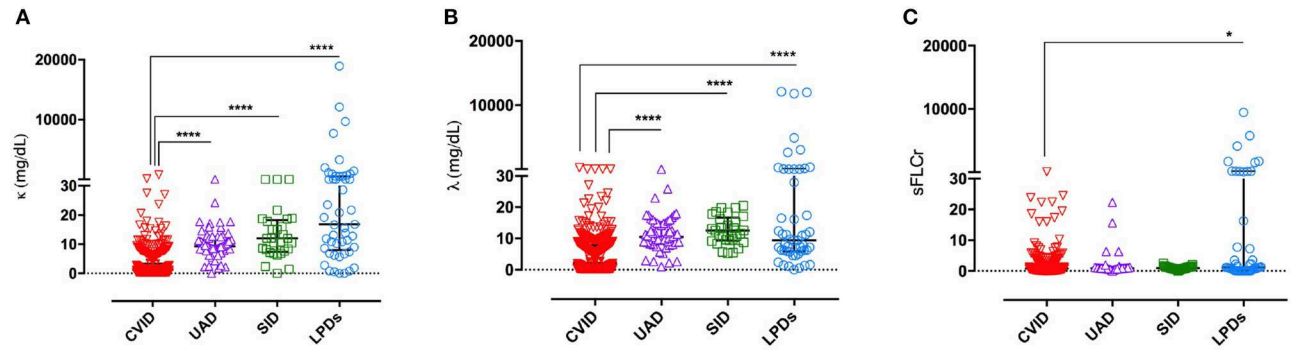

FIGURE 1 | sFLC levels in the differential diagnosis of hypogammaglobulinemia. CVID patients displayed lower of κ chains serum levels **(A)** and lower λ chains serum levels **(B)** in comparison to UAD, SID and LPDs groups. CVID had also lower FLCr in comparison to LPDs **(C)**. Individual values, median, and IQR are represented. Levels of significance for comparison with CVID group by u test: $^{*}P \leq 0.05$, $^{****}P \leq 0.0001$.

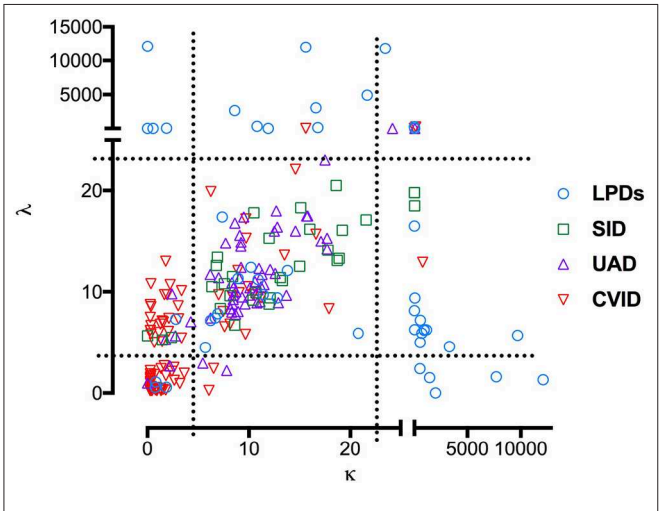

FIGURE 2 | Combined sFLC levels in the differential diagnosis of hypogammaglobulinemia. The scatter plot shows sFLC levels for 345 patients with CVID (red triangles), UAD (purple triangles), LPDs (blue circles) and non-hematological SID (green squares). Dashed lines represent reference for sFLC values.

($p < 0.0001$) of patients. LPD patients showed more commonly very high (>150 mg/L) concentration of at least one sFLC in comparison to CVID (45 vs. 0.6%, $p < 0.0001$). In the CVID, in addition to the above-mentioned patients who developed NHL, we recorded high concentration of both κ and λ chains in one patient with balanced FLCr and recurrent autoimmune cytopenia and chronic lymphadenopathy. None in the UAD and in the SID groups had very high concentration of κ or λ chains (**Figure 2**).

Sensitivity, Specificity, and Positive Predictive Value (PPV) of sFLC Pattern in CVID

Since 79% of CVID patients had at least one reduced FLC isotype but none of them had increased concentration of the other κ or λ chain, we defined as *CVID-like sFLC phenotype* the condition of having at least one reduced sFLC isotype without an increase of either κ or λ chain concentration, as previously suggested (15). In this cohort, sensitivity of the CVID-like pattern of sFLC was 78.3% (95% CI, 73.5 to 82.5%), and specificity was 87.6% (95% CI, 80.9 to 92.6%). By using this pattern as a diagnostic marker for CVID, the positive predictive value was 90.1% (95% CI, 91.0 to 96.1%), and the negative predictive value was 61.5% (95% CI, 56.5 to 66.4%).

Association Between sFLC Patterns and Clinical Phenotype in CVID Patients

Age at the time of enrollment did not significantly differ in CVID groups when considering the sFLC pattern. However, κ+λ+ patients were older at CVID diagnosis (45 y.o. IQR 32–45) in comparison to κ−λ− (38 y.o., IQR 32–45, *u*-test $p = 0.001$) and κ−λ+ group (39 y.o., IQR 26–42, *u*-test $p = 0.020$).

When analyzing the possible correlations between sFLC patterns and disease-related clinical phenotype and complications in CVID patients, the only disease phenotype significantly associated with the sFLC pattern was the "enteropathy" phenotype, with patients belonging to the κ−λ− group showing a higher frequency of enteropathy than those classified as κ+λ+ (18 vs. 8%, $p = 0.050$) (**Figure 3A**). In comparison to those included in the κ+λ+ and κ−λ+ group, κ−λ− patients were also more likely to have bronchiectasis (40 vs. 25%, $p = 0.030$ and vs. 20%, $p = 0.002$, respectively) and splenomegaly (55 vs. 33%, $p = 0.002$ and vs. 33%, $p = 0.001$, respectively) (**Figure 3B**). A medical history consistent with autoimmunity, cancer, lymphoma, or GLILD was not found to be associated with FLC patterns (**Figures 3A–D**).

Association Between sFLC Patterns and Laboratory Signatures of CVID

As shown in **Figure 4A**, the κ−λ− group showed lower IgG (2.6 g/L, IQR 1.4–3.7), IgA (0.1 g/L, IQR 0.1–0.2), and IgM serum levels (0.1 g/L, IQR 0.0–0.2 g/L) in comparison to κ−λ+ (IgG 3.5 g/L, IQR 2.3–4.6, *u*-test $p < 0.0001$; IgA 0.2 g/L, IQR 0.1–0.4, *u*-test $p < 0.0001$; IgM 0.3 g/L, IQR 0.1–0.5, *u*-test $p < 0.0001$), and κ+λ+ patients (IgG 3.4 g/L, IQR 2.8–4.3, *u*-test $p = 0.012$;

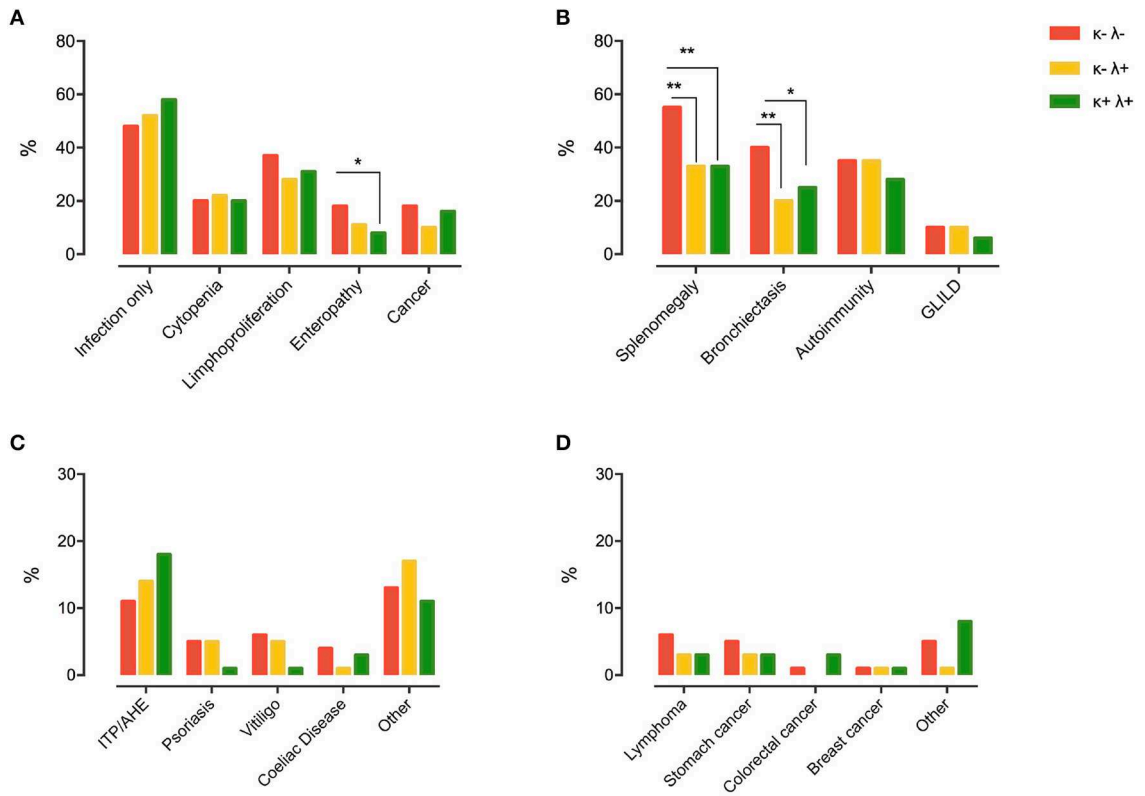

FIGURE 3 | Chapel clinical phenotyping **(A)** and CVID-related comorbidities **(B)** in participants grouped by FLC pattern (κ+λ− group was censored as it was composed of only nine subjects). The most prevalent types of autoimmune diseases and cancers in the different CVID subgroups are detailed in **(C,D)**, respectively. Levels of significance for comparison by Fisher's test: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. ITP, idiopathic thrombocytopenic purpura; AHE, autoimmune hemolytic anemia; GLILD, granulomatous and lymphocytic interstitial lung disease.

IgA 0.2 g/L, IQR 0.1–0.5, *u*-test $p < 0.0001$, IgM 0.3 g/L, IQR 0.2–0.6, *u*-test $p < 0.0001$). κ−λ− CVID patients also presented lower frequency of CD27+IgD–IgM– Switched Memory B cells (1.2%, IQR 0.4–5.0) when compared to both κ−λ+ and κ+λ+ groups (3.5%, IQR 1.0–5.9, *u*-test $p = 0.050$ and 2.5%, IQR 1.7–8.4, *u*-test $p = 0.027$, respectively) and the highest frequency of $CD21^{low}$ B cells among the CVID subgroups, with a significant difference in comparison to both κ−λ+ and κ+λ+ patients (9.0%, IQR 3.1–31.0 vs. 3.6, IQR 2.2–7.7, *u*-test $p = 0.013$, and *vs* 5.1%, IQR 1.9–6.5, *u*-test $p = 0.030$) (**Figure 4B**).

DISCUSSION

To our knowledge, this is the first multicenter study exploring the diagnostic value and the clinical association of sFLC pattern in a large cohort of CVID patients. Patients were recruited all over Italy, in six PAD referral centers. sFLC assay was performed in a single laboratory according to standard diagnostic procedure. As control groups, we enrolled subjects selected on the basis of a referral for hypogammaglobulinemia and finally diagnosed as UAD patients and secondary hypogammaglobulinemia, respectively. This latter group was further divided, on the basis of the final diagnosis, into a lymphoproliferative disease group (LPD), including MM, B-CLL, Non-Hodgkin Lymphomas and MGUS, and a secondary immunodeficiency group (SID) whose hypogammaglobulinemia was not related to LPDs.

In agreement with previous observations, we found that CVID patients tend to have a very low sFLC count with 79% of them presenting a significant reduction in one (almost exclusively κ) or both (κ and λ) chains, often to an undetectable level. All CVID patients were on IgRT; however, as previously shown, regular immunoglobulin administration did not affect the circulating sFLC concentrations (14–16). A very good intraindividual agreement between different sFLC assays was also registered.

Less than a half (41%) of the CVID patients presented an abnormal FLCr; for almost all of them this was the result of a decreased concentration of one chain with no increase in the other, therefore not consistent with a B cell malignancy. On the contrary, most patients (82%) in the LPD group showed an unbalanced FLCr due to an increase in either κ or λ chain, which was related to an underlying MM or B-CLL. In the SID and UAD groups a minimal percentage of unbalanced FLCr (3 and 12%, respectively) and no increase in κ or λ chain concentration were observed.

As suggested by Unsworth et al. our study supports the idea that in CVID patients, an impaired κ/λ ratio due to very low

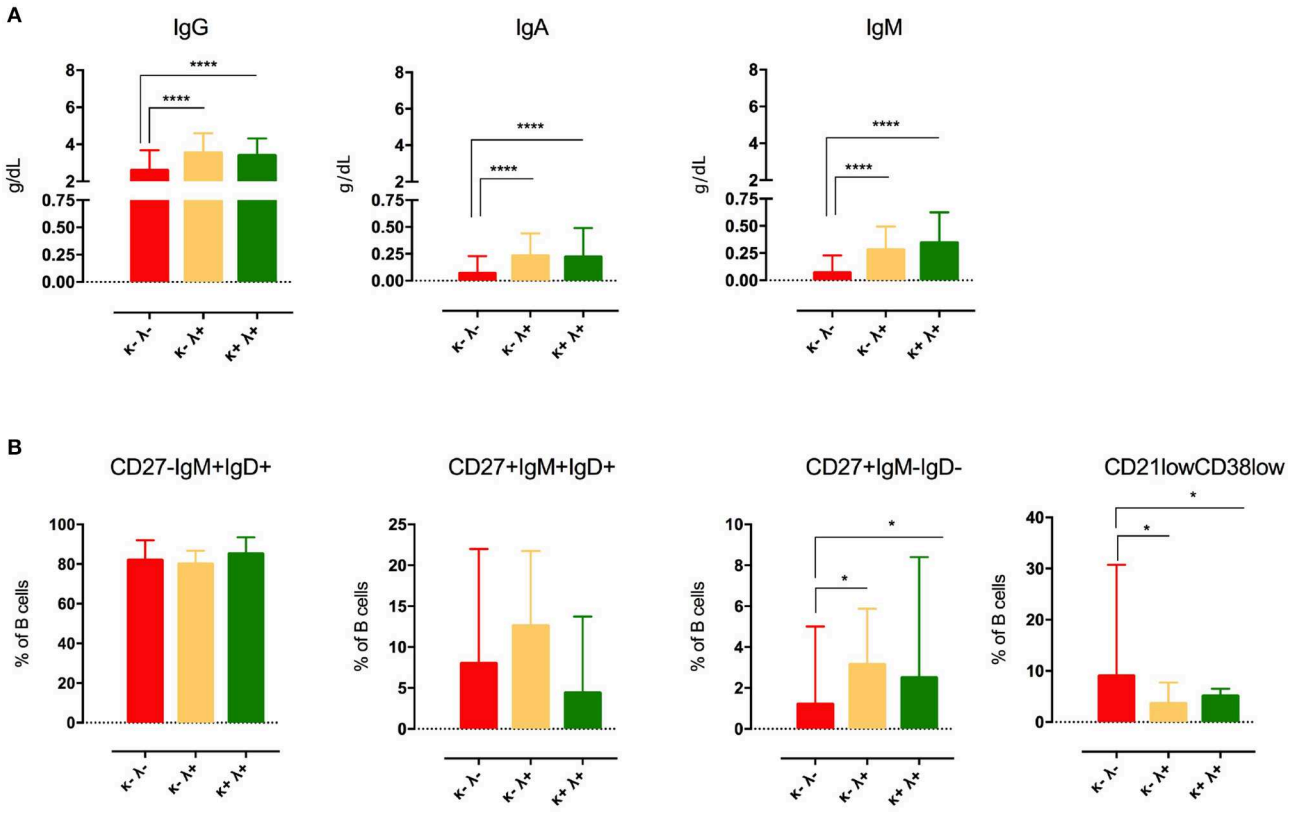

FIGURE 4 | Immunoglobulin serum levels at CVID diagnosis **(A)** and B-cell phenotype **(B)** in CVID patients grouped by sFLC levels (κ+λ− group was censored as it was composed of only nine subjects). Median and IQR are represented. Levels of significance for comparison with CVID group by u test: $^{*}P \leq 0.05$, $^{****}P \leq 0.0001$.

concentration of at least one sFLC most likely underlies the disease-related B-cell dysfunction, rather than a B lymphocyte clonality (14). On the other hand, the detection of an unbalanced sFLCr with a single light chain increase in two CVID patients from our cohort led to a diagnosis of a lymphoproliferative disease. One of these two patients, in particular, initially presented a reduction of both light chains and subsequently developed a significant increase of circulating lambda chain, with no detectable paraprotein and was eventually diagnosed a NHL. This suggests that sFLC assay might also be an effective tool in the follow-up strategy in a population with a well-known risk of hematological malignancies (4, 24, 25).

The overall percentage of CVID patients with at least one reduced light chain was similar to that reported by Compagno et al. (88%) and higher than described by Hanitsch et al. (63%). The so-called CVID-like sFLC pattern presented a sensitivity of 78.3% and a specificity of 87.6% and, as a diagnostic marker for CVID, a positive predictive value of 90.1% and a negative predictive value of 61.5% (15, 16). In the control groups the percentage of patients with at least one reduced light chain was 17, 10, and 30% for UAD, SID, and LPDs, respectively, with most of LPD patients showing a monoclonal increase in the other chain. In agreement with previous reports, our results confirm sFLC assay as a highly sensitive and specific tool in the diagnostic work-up of CVID that might be combined with other existing B cell assays. By considering also the SID group, not included in previous studies, we confirmed its reliability in "real life" differential diagnosis.

In our multicenter CVID cohort, the distribution of the four different patterns of sFLCs, normal kappa, and lambda chains (κ+λ+), reduced kappa chains (κ−λ+), reduced lambda chains (κ+λ−), and reduction of both chains (κ−λ−) confirms what was reported in our previous single-center study, and it shows similarities with another recently published study (15, 16). The more represented pattern in our cohort was indeed the (κ−λ−), with only few patients belonging to the (κ+λ−) group. In contrast to the paper by Hanitsch et al. we found that the number of patients in the (κ−λ+) group was more relevant, being slightly higher than the (κ+λ+) group.

The association of sFLC pattern with clinical data showed that CVID patients belonging to the κ−λ− group were more frequently classified as having unexplained enteropathy. We did not observe significant differences in terms of autoimmunity, GLILD, and cancer between the three groups. Moreover, κ−λ− patients presented more commonly bronchiectasis as described by Hanitsch et al. and splenomegaly as reported by Compagno et al. in comparison to those included in the κ−λ+ and κ+λ+ groups. Considering the infectious risk, when compared to the other groups, κ−λ− patients also showed lower serum IgG, IgA, and IgM levels at diagnosis, a profile that may predict a higher risk for bacterial infections and chronic lung damage (26). Thus, our findings point out to a more severe infectious phenotype as

the reason for the earlier diagnosis of CVID reported in κ−λ− patients. Moreover, we found a direct association between serum IgA levels and serum κ and λ chain concentrations in CVID and UAD cohorts (but not in LPDs and SID) and, in CVID only, a direct association between serum IgM and both serum κ and λ chain concentrations, further strengthening the correlation between sFLC levels and immunoglobulin production suggested by Unsworth et al. (14).

When considering circulating B cell subpopulations according to the EUROclass trial, we did not observe any difference in CD19+, transitional and marginal-zone B cells among the three main subgroups of CVID patients (20). However, κ−λ− CVID patients presented lower frequency of CD27+IgD–IgM– Switched Memory B cells when compared both to the κ−λ+ and κ+λ+ groups. This is consistent with the hypothesis that lower levels of sFLC may be an epiphenomenon of a higher degree of impairment in B cell differentiation, with a reduced B cell isotype switch leading to a lower level of immunoglobulin production. κ−λ− patients also showed the highest frequency of $CD21^{low}$ B cells between the CVID subgroups, with a significant difference if compared to both κ−λ+ and κ+λ+ patients. Of note, reduction in switched memory and increase in $CD21^{low}$ B cells, history of autoimmune cytopenia, presence of splenomegaly, and low serum IgA levels have been all suggested as potential predictors of GLILD (27, 28). According to the sFLC pattern, in our study κ−λ− patients present all the above-mentioned predictors apart from the history of autoimmune cytopenia. In contrast with Hanitsch et al. however, κ−λ− patients did not show significantly higher frequency of GLILD diagnosis, when compared to the other CVID subgroups (16). This difference might be partly due to the lack of universally recognized diagnostic criteria for GLILD, or simply to the different behaviors in case of GLILD suspicion at chest CT scan, particularly in asymptomatic patients. The choice between a more invasive approach with histologic confirmation and a watchful waiting strategy may indeed lead to a different degree of definite diagnoses. The ongoing prospective study in our cohort will hopefully grant more information on sFLC pattern as a possible long-term predictor of GLILD.

In conclusion, sFLC assay may be reasonably considered as a useful tool to be included in the differential diagnosis of antibody deficiencies, with a good specificity and sensitivity for CVID. Due to its well-known role in diagnosis and prognosis of LPDs, and based on our and previously published data, we suggest adding sFLC dosage to the initial screening of hypogammaglobulinemia and during follow-up. The assay may help in distinguishing CVID from other causes of hypogammaglobulinemia, in combination with other tests, and it offers the chance of early detection of monoclonal lymphoproliferation occurring over years. Moreover, the sFLC pattern appears to be related to other laboratory parameters and to disease phenotypes and clinical manifestations of CVID. If our data will be confirmed by further and long-term follow-up studies, sFLC assay will thus be considered a useful prognostic tool that, in combination with other instruments, might help in identifying disease phenotypes at higher risk to develop enteropathy and chronic lung damage or splenomegaly and further complications, thus designing a more personalized follow-up strategy for CVID patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico per la Sperimentazione Clinica della Provincia di Padova. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS and FP wrote the paper and contributed to patient enrolment and data collection. FP did the statistical analysis. FC and CMi equally contributed to designing and coordinating the study, were involved in patient enrolment and data collection, manuscript writing, and revision (joint authorship). CA, IQ, GS, AM, AVa, and MD contributed to designing the study, were involved in data analysis, and revised the paper. NC contributed to designing the study and to patient enrolment. AP, CMa, AVu, FV, and RR contributed to patient enrollment, data collection and paper revision. DF contributed to data interpretation, and manuscript preparation and revision. SA performed the sFLC assay and contributed to manuscript preparation and revision. MP contributed to manuscript preparation and revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00319/full#supplementary-material

Figure S1 | Linear correlation between IgA serum levels at diagnosis and serum κ **(A)** and λ **(B)**. IgA levels were directly associated with free κ and λ values in CVID and UAD, but not in LPDs and SID.

Figure S2 | Linear correlation between IgM serum levels at diagnosis and serum κ **(A)** and λ **(B)**. Only in the CVID cohort IgM was directly associated with both serum κ and λ chain concentrations. No association was found between sFLC and IgM levels at diagnosis in the control groups.

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