CONTEMPORARY CHALLENGES IN DIAGNOSIS AND TREATMENT OF PREDOMINANTLY ANTIBODY DEFICIENCY

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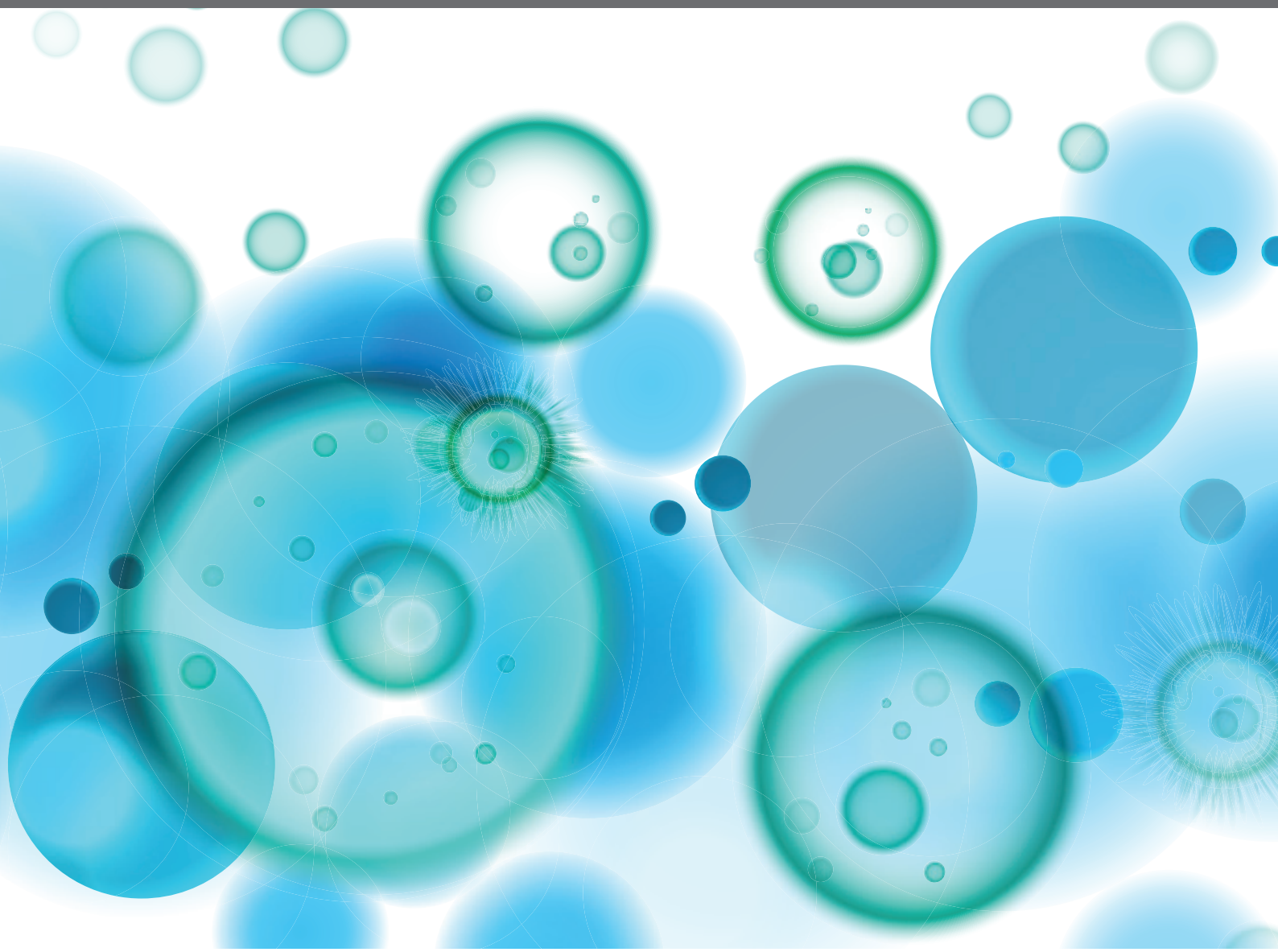

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CONTEMPORARY CHALLENGES IN DIAGNOSIS AND TREATMENT OF PREDOMINANTLY ANTIBODY DEFICIENCY

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Editorial: Contemporary challenges in diagnosis and treatment of predominantly antibody deficiency

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Editorial on the Research Topic

Contemporary challenges in diagnosis and treatment of predominantly antibody deficiency

Primary immunodeficiencies (PID) otherwise referred to as Inborn Errors of Immunity (IEI) are rare inherited diseases of the immune system. According to the International Union of Immunological Societies (IUIS) classification, predominantly antibody deficiency (PAD) is the most common PID, characterised by low serum immunoglobulin levels, poor vaccination responses and a high incidence of infectious and non-infectious complications including cancer. In adults, IgA deficiency (IgAD) and Common Variable Immunodeficiency Disorders (CVID) comprise the majority of these conditions. Diverse clinical, immunological, and genetic phenotypes in PAD result in diagnostic delays and poor access to targeted treatments, accounting for the early mortality and high morbidity of this population. The current series of fourteen articles on Contemporary Challenges in Diagnosis and Treatment of PAD explores aspects of the diagnosis and treatment of of this group of conditions. Here, the editors of this section summarise the main findings of the articles in this series.

Diagnosis and semantics

The first article in the series explores the terminology and semantics of this group of disorders (Ameratunga et al.) In recent years there has been a move to rename these conditions as Inborn Errors of Immunity (IEI) rather than PIDs. However, the majority of patients with PIDs do not yet have a genetic explanation. These disorders include CVID (by definition), IgAD, transient hypogammaglobulinemia of infancy (THI) and Good's syndrome comprising the majority of PIDs. Additionally, not all patients with conditions such as Severe Combined Immunodeficiency have a genetic diagnosis. Therefore, this article argues that at present it is premature to label all patients with PIDs as having IEIs. In the future, diagnostic advances may make it possible to genetically define all of these conditions and then the use of PID and IEI will be interchangeable.

Where feasible, all patients with PIDs should be offered genetic studies. There are many overlapping clinical advantages in securing a genetic diagnosis. It may allow the accurate diagnosis of an atypical presentation of PID, which could permit prenatal or pre-implantation diagnosis, direct prognostic monitoring of patients e.g. monitoring for development of non-infectious complications associated with a particular genetic defect, or improve patient access to targeted therapeutics. In PAD, genetic diagnosis rates are currently low (<20-30% in most nonconsanguineous cohorts). Despite the aforementioned genetically undiagnosed PAD patients, the use of Next Generation Sequencing (NGS) has significantly increased the number of genes shown to underlie PAD. The article by Rojas-Rostrepo et al., presents the findings of NGS in 291 patients with PAD. In 24.7% (72/291) of patients a relevant genetic defect was identified by NGS. It should be noted that some of these patients had gene panels while others had whole exome sequencing. The authors confirm the yield from this study may have been higher if whole exome sequencing had been deployed for all patients, showing the validity of this approach in the diagnostic work up of PAD. A wide variety of genetic mutations were identified, highlighting the genetic complexity of these diseases. Importantly, an impact on protein expression and/or function was proven in these patients (n=72), confirming variant pathogenicity. It should be noted that the diagnostic yield in consanguineous populations is much higher for predominantly autosomal recessive conditions.

In PAD, vaccine responses are characteristically low, and notoriously difficult to assess in patients receiving immunoglobulin replacement therapy. The study by Hansen et al., describes a new approach to assess responses to the pneumococcal vaccine. Currently at least five criteria for assessing responses to Pneumovax® exist. This article examines the use of a Z score to quantify responses to the Pneumococcal vaccine. The advantage of the Z score is that it avoids the dichotomous responses advocated by the American Academy of Asthma Allergy and Immunology. Such an approach distinguishes patients with symptomatic hypogammaglobulinemia from those with milder symptoms. Future studies will indicate if such an approach resolves difficulties with interpreting vaccine challenge responses in antibody deficient patients.

There is often a long lag between the onset of symptoms and identification of hypogammaglobulinemia in antibody deficiency disorders. The study by Piza et al., explores the utility of cost-effective serum electrophoresis and calculated globulin fractions for identification of patients who may have hypogammaglobulinemia. The area under the curve in the gamma region of serum protein electrophoresis (EPG) may prove to be a useful screening tool for earlier diagnosis of hypogammaglobulinemia. This may reduce the diagnostic lag to implement quicker treatment for patients.

Mechanism of disease

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) regulate cellular immunity and represent important targets for immunotherapy. The article by Hao and Cook explores interesting parallels between phenocopies and patients with genetic defects in CTLA-4 or PD-1. Anti-CTLA-4 treatment is used to treat patients with severe autoimmunity and malignancy, whilst checkpoint inhibitors including anti-PD-1 are used to treat cancer, most notably metastatic melanoma. Patients suffering from genetic defects of CTLA-4 and PD-1 are at risk of autoimmunity. The recapitulation of the phenotype by drug treatment confirms the role of these molecules in the pathogenesis of these disorders. There are however interesting differences in the phenotype of CTLA-4 haploinsufficiency, and patients treated with ipilimumab (anti CTLA-4). Most notably patients with CTLA-4 haploinsufficiency had a much greater risk of infections. Similarly, a family with congenital deficiency of PD-1 had similar autoimmune manifestations including type 1 diabetes, as is often seen in patients undergoing treatment with PD-1 checkpoint inhibitors. Some of the differences between the congenital versions of these disorders and their phenocopies may relate to incomplete inhibition of the pathway and any effect of the Fc component of the antibody used to block the molecule.

Kabir et al., review what is known about Good's syndrome, a rare, combined immunodeficiency. This disorder is characterised by hypogammaglobulinemia, B cell lymphopenia and a T cell defect in the presence of a thymoma. Moreover, many features overlap with CVID including bacterial infections, and haematological disease, as well as an absence of a genetic explanation for disease. Thus, the authors suggest a more thorough investigation of Good's syndrome is required to understand the underlying immunopathology and to support a more definitive diagnosis.

The impact of COVID-19, including vaccines on antibody deficient patients

The COVID-19 pandemic has caused a global crisis. Apart from well over 6 million deaths, large numbers of individuals are suffering from ongoing physical and psychiatric morbidity. In addition, it has raised significant concerns for patients with PAD due to poor immunity to vaccination and increased susceptibility to severe infection with pathogens including viruses. Thus far vaccination and antiviral therapeutics represent the safest options for patients with normal immune systems as well as those suffering from antibody (and other) immunodeficiencies, with triple doses of vaccination recommended as a primary regimen for those with immunodeficiencies (compared to a double primary dose for healthy individuals). Furthermore, it is expected that intravenous or subcutaneous immunoglobulin (SCIG/IVIG) replacement therapy will provide added protection for those antibody deficient recipients.

Ameratunga et al., review the potential utility of vaccines in patients with antibody deficiency. Two recent studies have shown that patients with CVID are able to generate antibodies against tetanus toxoid and Haemophilus influenza type B. This indicates that COVID-19 vaccines will be at least partially effective in patients with CVID. However, the breadth, longevity, and protective capacity of different facets of immunity: antibodies, memory B cells and memory T cells is required to understand whether vaccine-elicited responses to the SARS-CoV-2 virus can protect from breakthrough infection and severe disease. This article highlights the need for individual assessment of patients to determine the degree of immunity generated in response to vaccination, especially in patients receiving SCIG/IVIG where antibody responses cannot be accurately quantified. Moreover, it might be necessary to boost patients without a measurable SARS-CoV-2-specific T cell response every six months, to ensure longevity of the response. However, this will need to be closely monitored to prevent overactivation and exhaustion of the T cell compartment.

In line with this, the article by Quinti et al. explores antibody and cellular responses to COVID-19 vaccines. It appears that patients with CVID generate atypical memory B cells in response to COVID-19 vaccines, which might be short-lived and only develop partial T cell immunity. The authors suggest robust immunity can be induced with post infection-induced vaccination. In contrast, infection produces typical memory B cells, which may be more protective. Here, the authors suggest CVID COVID-19 survivors should have booster vaccines as hybrid immunity may provide long-lasting protection. Furthermore, since reinfection with SARS-CoV-2 has been reported the authors postulate that CVID patients may benefit from preventive therapies such as administration of monoclonal antibodies against the spike protein of SARS-CoV-2.

The response to COVID-19 vaccines in patients with mild antibody defects has not been studied. The study by Sauerwein et al., evaluated antibody and $CD4^+$ T cell responses after two doses of the Pfizer BNT162b2 vaccine in 31 adult PAD patients. 76% PAD patients mounted protective SARS-CoV-2-specific IgG responses whereas 87% generated SARS-CoV-2-specific IgA antibodies. The study also showed activation of $CXCR5^+$ $CD4^+$ T cells after vaccination. Activation of these follicular helper $CD4^+$ T cells was associated with the generation of anti-Spike antibodies. It appears patients with mild antibody defects respond to COVID-19 vaccines analogous to normal controls. Patients suffering from CVID had variable $CD4^+$ T cell responses, which correlated with the levels of neutralising antibody titres, suggesting an important role for $CD4^+$ T cells in vaccine efficacy in CVID. This study attests to the importance of being able to measure T cell responses to COVID-19 and supports that at least two doses of the Pfizer BNT162b2 vaccine were required for vaccine efficacy in this population.

Complications of antibody deficiency

Infection is the most common complication in PAD. Viral infections are frequently the source of morbidity and mortality in immunodeficient patients. In contrast to the broad array of antibacterial drugs, there are fewer effective antiviral drugs. Some infections such as intractable norovirus can lead to death from nutritional complications. A case series by Chan et al., highlights the role of Cytomegalovirus (CMV) in producing chronic infection in patients with CVID. This cohort had a high mortality attesting to the importance of CMV infections in prognosis and CMV centric therapeutics in patients at risk of CMV-related disease.

Furthermore, an increased risk of malignancy in patients with antibody defects has been identified for several decades. These can either arise spontaneously or following a viral infection. It has been recognised for many years that patients with antibody defects have an increased susceptibility to gastric cancer as well as lymphoid malignancy. Thus, the mechanisms underlying cancer susceptibility are urgently needed for better diagnosis and treatment of this comorbidity. In genetically undiagnosed patients, the study by Bruns et al., shows that 12.3% (27/219) patients had cancer, with gastric cancer, non-Hodgkin's lymphoma and non-melanoma skin cancer being the most prevalent. Whilst no significant differences in immunological phenotype were observed, a definite or likely genetic diagnosis was identified in 11% of the patients with cancer. Furthermore, it was posed that a likely genetic susceptibility to cancer was identified in 14.3% of patients. This provides important insight into the prevalence of cancer in PAD and the genetic basis of disease, which may contribute to genetic screening for this comorbidity. In contrast, the review by

Abolhassani et al., examined the prevalence of cancer in the context of specific monogenic defects. The complex development of cancer was compared between PAD patients and other PID groups, based on 10 hallmarks of cancer. This provides information, which can improve the genetic diagnosis and identification of hallmarks of PAD, which may direct more targeted patient treatment.

Patients with CVID are predisposed to liver disease, most notably nodular regenerative hyperplasia (DiGiacomo et al.). The measurement of transient liver stiffness in patients with CVID is a non-invasive method for identification and monitoring this complication. This study by DiGiacomo et al. shows that this test can be undertaken periodically to diagnose and monitor progress of portal hypertension in these patients to prompt referral to the hepatology clinic.

Therapy

There is ongoing debate about the merits of subcutaneous vs intravenous immunoglobulin therapy in antibody deficiencies. This article explores patient preferences for SCIG vs IVIG (Gonzalez et al.). It appears there is considerable heterogeneity of preferences in the route of administration. This suggests an integral partnership between physician and patient will enhance the therapeutic relationship, to improve patient care.

Summary

The past decade has provided improved insights into the molecular and immunological underpinnings of PAD. The articles in this series have covered many areas of ongoing research attesting to the number of groups around the world investigating PAD. This augurs well for patients suffering from these conditions as new diagnostic and therapeutic modalities will result from these endeavours. This will ultimately increase diagnostic rates, reduce the diagnostic delay, and improve patient access to targeted therapies leading to improved patient quality of life and reducing the morbidity of disease.

Authors contributions

RA and ESJE reviewed the published papers on the Research Topic and wrote the manuscript. All authors critically evaluated, edited, and approved the submitted article.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPINION
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Are All Primary Immunodeficiency Disorders Inborn Errors of Immunity?

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INTRODUCTION

It is almost 70 years since the first description of Bruton's agammaglobulinemia (1). In the last decade there has been a rapid increase in the rate of discovery of new genetic defects in primary immunodeficiency disorders (PIDs), largely due to the advent of Next Generation Sequencing (NGS) (2, 3). NGS utilises massively parallel sequencing to analyse either the exome (WES) or the entire genome (WGS). The International Union of Immunological Societies (IUIS) expert committee of the WHO has curated over 400 such disorders (2).

This sequencing revolution has had profound benefits (and some disadvantages) for patients and their clinicians as well as scientists seeking to identify new disorders (4, 5). As termed by Robert Good, these "experiments of nature", have offered unique scientific insights into functioning of the immune system (6).

The many overlapping benefits of genetic confirmation for patients include certainty of diagnosis, prognostic insights and specific treatments (5). It has ushered in the era of personalised medicine. Identification of a gain for function mutation of *PIK3CD* for example, may lead to specific treatments such as idelalisib in addition to subcutaneous or intravenous immunoglobulin (SCIG/IVIG) replacement.

With the rapid increase in the discovery of new genetic defects, there has been a move to name these conditions inborn errors of immunity (IEI) (2). The Merriam Webster dictionary states Inborn is "being a part of the innermost nature of a person or thing" and synonyms include *congenital*, *hereditary* and *inherited*. Inborn thus implies these conditions are genetic and inherited, which will be transmitted to future generations. Errors in this context infer mutations, which are pathogenic and underlie the phenotype of the patient.

Although there is an argument for changing the name from PIDs to IEI, or using these terms interchangeably, there are several caveats. Three of the most common conditions, which numerically comprise the majority of patients with PIDs, do not currently have a definable genetic basis; IgA deficiency (IgAD), Common Variable Immunodeficiency Disorders (CVID) and Transient

Hypogammaglobulinemia of Infancy (THI). Other well-recognised conditions, which do not have a genetic explanation at this time, include Good's syndrome and CD4 lymphopenia. Even within well-defined phenotypes such as agammaglobulinemia with absent B cells or Severe Combined Immunodeficiency (SCID), not all patients have a genetic explanation for their disorder (**Figure 1**). This is a perspective on why these terms are not currently interchangeable and why it may be premature to abandon the term PID in favour of IEI (**Table 1**).

IGA DEFICIENCY

IgA deficiency (IgAD) is the most common PID in humans. IgAD can occur in the context of other well-defined PIDs such as SCID or X-linked hyper IgM syndrome or as a sporadic disorder. The frequency of sporadic IgAD may be as high as 1:358 in blood donors (7). The majority of patients with sporadic IgAD are asymptomatic, presumably because other arms of the immune system can compensate for the lack of IgA in serum and on mucosal surfaces. The genetic basis for sporadic IgAD is not understood (13). While there is an increased proportion of IgAD patients with *TNFRSF13B*/TACI mutations, the prevalence of such variants exceed that of sporadic IgAD (14). Application of the American College of Medical Genetics (ACMG) criteria indicates TACI mutations are not the cause of sporadic IgAD (15).

COMMON VARIABLE IMMUNODEFICIENCY DISORDERS

CVIDs are the most common symptomatic PID in adults and children. The prevalence of CVID varies between 1:25 000 to less than 1:100 000 in the general population. Although there may be ascertainment bias, these disorders are less common in Asian and African populations. Reports from developed Asian countries with advanced healthcare systems, such as Taiwan, Japan or South Korea indicate a very low frequency of CVID (16–19). In contrast, a recent study has shown an unexpected high prevalence of CVID in the indigenous Māori of New Zealand (20). The basis for these ethnic-specific rates is not known.

By definition, the genetic causes for CVID are unknown (21–23). There are now over forty genetic variants which are associated with CVID (3, 8, 24). Some of these are causative (*NFKB1, NFKB2* etc) while others predispose to or modify disease severity (*TNFRSF13B*/TACI, *TNFRSF13C*/BAFFR, *MSH5* etc.) in CVID. Sometimes mutations in genes such as *RAG* can lead to atypical presentations which might be considered CVID-mimics. If patients have a causative genetic defect, they are now deemed

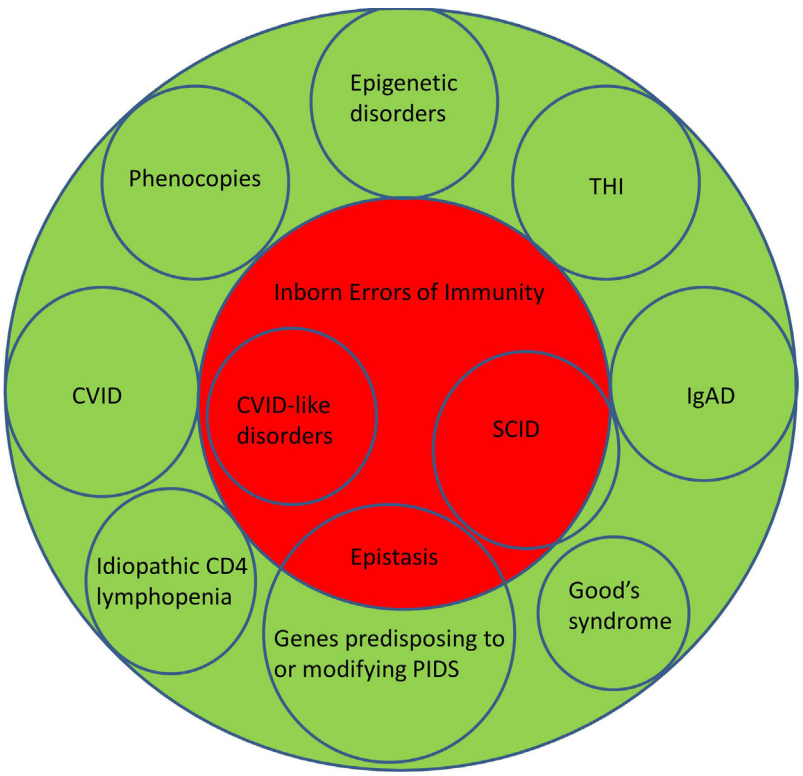

FIGURE 1 | Illustrating the relationship between Primary Immunodeficiency Disorders (green) and Inborn Errors of Immunity (red). CVID, Common Variable Immunodeficiency Disorders; IgAD, IgA deficiency; SCID, Severe Combined Immunodeficiency; THI, Transient Hypogammaglobulinemia of Infancy.

TABLE 1 | PIDs for which a causative genetic basis has not been identified in all patients.

Disorder	Genetic basis	Comment
IgA deficiency	Unknown	Commonest PID (7)
CVID	Unknown (by definition)	Patients with causative genetic defects are reclassified as CVID-like disorders (3, 8)
THI	Unknown	Retrospective diagnosis (9)
Idiopathic CD4 lymphopenia	Unknown	Increasingly recognised with the advent of new-born screening (10)
Good's syndrome	Unknown	Thymic abnormalities may contribute to the combined immunodeficiency (11)
SCID (and many other similarly well- characterised disorders)	Mostly known	Not all patients with a SCID phenotype have an identifiable mutation
Phenocopies	Acquired disorders	Can be identified by genetic sequencing (2)
Epigenetic defects	Acquired disorders	Difficult to identify (12)

CVID, Common Variable Immunodeficiency Disorders; SCID, Severe Combined Immunodeficiency; THI, Transient Hypogammaglobulinemia of Infancy.

to have a CVID-like disorder with their own specific mutation. Patients with CVID-like disorders could be considered to have an IEI (**Figure 1**).

In consanguineous societies, the proportion of patients with CVID-like disorders is approximately 60-70% (25, 26). Patients from such countries have highly penetrant autosomal recessive monogenic disorders often presenting in childhood. In contrast, only 25% patients from non-consanguineous populations have an underlying causative mutation, mostly late-onset autosomal dominant disorders (27). In non-consanguineous societies, this leaves 75% of CVID patients without a genetic explanation and IEI would seem an incorrect term for these patients.

CVID-like disorders are characterised by marked genetic, allelic and phenotypic heterogeneity (8). It is apparent that several causative genetic defects lead to the same phenotype of recurrent infections and autoimmune disorders (locus heterogeneity, genocopy). Similarly, there can be marked phenotypic variation in the same family carrying the identical mutation. In one of the three families where mutations of *NFKB1* were first described, one brother was completely asymptomatic, while his sister passed away from late onset combined immunodeficiency (LOCID). Other members of the family had predominantly autoimmune disorders (28, 29). This phenotypic heterogeneity of CVID-like disorders is compatible with IEI as the majority of patients with causative genetic defects would be expected to become symptomatic at some stage of their lives. It is likely environmental triggers such as Herpes simplex infections may alter their prognostic trajectory.

To complicate the genetics of CVID and CVID-like disorders, a group of genes predispose to or modify disease severity in CVID and CVID-like disorders (30). These genes include *TNFRSF13B*/TACI, *TNFRSF13C*/BAFFR, *MSH5* etc. While there is a higher prevalence of mutations of these genes in patients with CVID and CVID-like disorders, their frequency in the general population far exceeds that of CVID (31). The majority of healthy individuals carrying these mutations will not suffer CVID. While these could be considered variants and are inherited, they are not causative and should not be considered IEI. The ACMG criteria cannot be applied to genes which predispose to or modify CVID (15).

This was recently illustrated in a family presenting with a severe CVID-like disorder (32). The proband had mutations of both *TNFRSF13B*/TACI as well as *TCF3*. Family studies showed it was the *TCF3* mutation, which segregated with the CVID-like disorder, while the *TNFRSF13B*/TACI mutation modified the disorder in an epistatic fashion (33). Epistasis is the synergistic interaction of two or more genes which can modify disease severity or lead an entirely different phenotype (34). This family illustrates the complexity of CVID and CVID-like disorders and the need for accurate description of the underlying genetics. Individuals from this kindred carrying the *TCF3* mutation could be considered to have an IEI but not those with *TNFRSF13B*/TACI, which modified the severity of the disorder.

TRANSIENT HYPOGAMMAGLOBULINEMIA OF INFANCY

Transient hypogammaglobulinemia of infancy is an important cause of reduced immunoglobulin levels in early life (35). Current thinking is that there is delayed maturation of IgG production in these infants. By definition, the IgG normalises over time and THI is a retrospective diagnosis. Recently it has been shown that the majority of patients do not normalise their IgG until after four years of age (9).

The genetic basis of THI is currently not understood. This is likely to be a common PID but may not be recognised as many patients are asymptomatic. Again, it is inaccurate to term these infants as having an IEI.

OTHER IDIOPATHIC DISORDERS WITH A PROBABLE GENETIC BASIS

There are many other well-defined disorders where the genetic basis has not been identified. This includes patients with Good's syndrome, a combination of thymoma, CD4 lymphopenia and hypogammaglobulinemia (11). Most patients with Good's syndrome are diagnosed following a CT scan of the thorax, when the thymoma is identified.

Following the advent of new-born screening it has become apparent many patients with low numbers of T cell receptor excision circles (TRECs) have idiopathic T cell lymphopenia

rather than Severe Combined Immunodeficiency (SCID) (10). The long-term prognosis of these infants is not known. The genetic basis for persistent idiopathic T cell lymphopenia is not understood and it would not be accurate to label this condition as an IEI (36).

PHENOCOPIES

The WHO/IUIS committee has recognised a group of disorders called phenocopies, which do not easily fit with IEI (2). Phenocopies are caused by discrete clones of cells, which proliferate and reproduce the phenotype of the disorder. There are many examples of these somatic disorders including the autoimmune lymphoproliferative syndrome (ALPs). While these conditions have a genetic basis, they cannot be inherited and do not meet the inborn component of IEI. It may still be appropriate to call them PIDs as there is no secondary cause.

EPIGENETIC DISORDERS

Monozygotic twins discordant for CVID have been described (12). The authors speculated the explanation was epigenetic changes in several genes including *TCF3*. It will be important to show these methylation patterns are stable over time and segregate with the phenotype of the affected twin. It seems likely other PIDs will be identified in the future, where epigenetic changes are the basis of the disorder. Epigenetic changes are not considered IEIs but would be considered PIDs.

DISCUSSION

This essay has examined the interchangeability of the terms PID and IEI. There are many reasons why the term primary immunodeficiency disorder is currently preferred to inborn error of immunity. This is not merely a matter of semantics, as it has important implications for scientific accuracy as well as clinical management of these patients.

Unlike IEI, PID does not necessarily imply there is an inherited genetic basis for the disorder and could include phenocopies. The aberrant clone of cells harbouring the mutation is directly responsible for the clinical manifestations of disease. In secondary causes such as lymphoma, the immune defect is consequent to the malignant process. Phenocopies can thus be considered PIDs as they are the cause of the disorder, but not IEIs as they cannot be inherited despite having a genetic basis.

Epigenetic disorders cannot be easily identified by current sequencing technologies but may be more prevalent than is currently perceived. If more disorders are shown to have an epigenetic basis, the term IEI will be inappropriate as these conditions cannot be inherited. They will however remain PIDs as there is no secondary cause for the disorder.

Scientific integrity is important. Given that the genetic basis of CVID is by definition not known, stating patients with CVID have an IEI is inaccurate. In addition to scientific inaccuracy, semantics can also adversely affect patient care. As seen in the description of CVID and CVID-like disorders, the genetics of PIDs are complex (3, 24, 37). It is very important for patients to be appropriately counselled, particularly in non-consanguineous populations that there is a greater chance a causative mutation will not be identified. Such genetic counselling is an essential part of pre-analytical testing. If patients with CVID are advised they have an IEI, it may create unrealistic expectations that a causative genetic defect will be identified.

Furthermore, it may create anxiety as IEI more than PID might indicate the disorder will be passed to the next generation. PIDs, like many familial conditions are associated with parental guilt. This may be exacerbated if the genetic basis is not identified after testing. PIDs in contrast do not imply all patients have a genetic basis for their disorder, as there are other pathogenic mechanisms. Classifying CVID as a subset of PIDs is both scientifically accurate and is more helpful in managing patient expectations during pre-analytical counselling (**Figure 1**).

IEI also implies greater scientific understanding than is currently the case for many disorders. Asymptomatic IgAD likely comprises the majority of patients with PIDs. As noted above, the genetic basis for sporadic IgAD is unknown. As with CVID, it is scientifically more accurate to term these conditions PIDs than IEIs. Numerically, symptomatic and asymptomatic patients with sporadic IgAD far exceed all other PIDs and cannot be termed IEIs.

Similarly, although rare familial cases of THI have been described in siblings, the vast majority are sporadic (9). The genetic basis for familial THI is unknown. The use of the term IEI could cause unnecessary parental anxiety and guilt if the index child has suffered invasive infections and has needed SCIG/IVIG. In this context IEI implies a greater understanding of THI than is the case.

One argument for using IEI is the case of gain of function (GOF) mutations leading to autosomal dominant disorders. Such heterozygous cases lead to a phenotype, which is often very different from patients with loss of function mutations in the same gene. Although at first glance, the term PID may not seem to fit well, these conditions could be considered to be an immunodeficiency of regulatory elements of gene function. A similar argument could be made for patients with CVID-like disorders presenting with autoimmunity with a minimal history of infections.

Inspite of these differences, there are however areas of agreement between the terms PID and IEI. Both exclude secondary causes such as infections, gut disease, malignancy, renal immunoglobulin loss etc. (38). Generally secondary causes such as malignancy can be easily distinguished from PIDs. The age of the patient is helpful in considering the likely secondary causes. The passage of time and family history can usually help distinguish secondary causes from a primary disorder.

In the last decade, there has been rapid progress in the identification of the genetic basis of many PIDs. If there is

another future genetic revolution akin to NGS, it is possible all patients with PIDs will have a genetic diagnosis. Apart from patients with phenocopies and epigenetic changes, the two terms PID and IEI may be interchangeable.

All current definitions of CVID exclude a known cause for hypogammaglobulinemia (21–23). Counterintuitively, if the genetic basis of CVID was understood in all patients, the disorder will cease to exist (8). All such patients with CVID-like disorders will have their own genetic defect/IEI. It would seem reasonable to revisit this topic periodically, particularly if there have been major advances in technology.

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RA wrote the first draft. All authors contributed to the article and approved the submitted version.

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REVIEW
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Hallmarks of Cancers: Primary Antibody Deficiency *Versus* Other Inborn Errors of Immunity

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Inborn Errors of Immunity (IEI) comprise more than 450 inherited diseases, from which selected patients manifest a frequent and early incidence of malignancies, mainly lymphoma and leukemia. Primary antibody deficiency (PAD) is the most common form of IEI with the highest proportion of malignant cases. In this review, we aimed to compare the oncologic hallmarks and the molecular defects underlying PAD with other IEI entities to dissect the impact of avoiding immune destruction, genome instability, and mutation, enabling replicative immortality, tumor-promoting inflammation, resisting cell death, sustaining proliferative signaling, evading growth suppressors, deregulating cellular energetics, inducing angiogenesis, and activating invasion and metastasis in these groups of patients. Moreover, some of the most promising approaches that could be clinically tested in both PAD and IEI patients were discussed.

Keywords: inborn errors of immunity, primary immunodeficiency, predominantly antibody deficiency, hallmarks of cancer, immune dysregulation, genome instability, chronic inflammation

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INTRODUCTION

Inborn Errors of Immunity (IEI), formerly known as primary immunodeficiencies, comprise at least 450 inherited diseases, from which selected patients manifest a frequent and early incidence of malignancies (1–3). As the main presentation, IEI patients are prone to recurrent infections (due to bacterial, viral, and parasitic agents) that predispose individuals to a chronic increase in inflammatory mediators, contributing to neoplasia (e.g., reactive oxygen and nitrogen intermediates, prostaglandins, and inflammatory cytokines). The longer the inflammation persists due to inadequate or inappropriate treatments, the higher the risk of associated tumorigenesis and the survival advantage of a cancerous cell (4). However, several other intrinsic and extrinsic causes of malignancies have been identified in both IEI-associated hematologic and solid tumors (5, 6). Considering the heterogeneous pathogenesis of IEI, different mechanisms underlying tumorigenesis in these patients would be expected. From an oncologic point of view, the main hallmarks of cancer have recently been suggested to dissect the complexity of neoplastic disease (7). The presented review compares oncologic hallmarks and the molecular defects underlying primary antibody deficiencies (PADs) with other IEI-associated cancers. The current published literature collection highlights that PAD patients have more diverse hallmarks of cancers

compared to other IEIs (except combined immunodeficiency and immune dysregulation) and have a higher number of cases with heterogeneous genetic defects or unknown molecular etiologies. Of note, several therapeutic options are currently available for these diverse pathogeneses in PAD patients with cancer susceptibility, which should be considered by clinical immunologists and treating physicians.

AVOIDING IMMUNE DESTRUCTION

The ability of recognition and elimination of developing tumors in the absence of external therapy, which is known as cancer immunosurveillance, can be defective in certain types of IEIs (8, 9). Although the overall increased relative risk of cancer in IEI patients is less than twofold, a skewed spectrum of cancers (mainly lymphoid malignancies in males) can result from different gene defects (10). Innate and adaptive cytotoxicity against pre-malignant or malignant cells can be affected by mutations associated with dysfunction of natural killer (NK) and $CD8^+$ T cells (1). Therefore, the intrinsic genetic defects affecting the development or function of T cell (presenting with combined immunodeficiency, major histocompatibility complex class I deficiency, or hyper IgE syndromes) and NK cell (GATA2, MCM4, and FCGR3A deficiencies) may lead to cancer, in particular carcinomas (11–15). Familial hemophagocytic lymphohistiocytosis patients with mutations in *UNC13D, PRF1, STXBP2*, and *STX11* also present a significant defect in cytotoxicity, causing lymphoproliferative diseases and oncogenesis (16, 17).

Moreover, a proportion of patients with diseases of immune dysregulation show an increased susceptibility to herpes virus infections (mainly Epstein–Barr virus [EBV]-induced lymphoproliferative complications and malignancies), which resulted from defects in co-stimulatory molecules essential for $CD8^+$ memory T-cell formation (e.g., CD27 and CD70 deficiencies and OX40 deficiency associated with higher risk of lymphoma and sarcoma) (18–21). Several other genes coordinate $CD8^+$ T-cell activation and memory generation *via* various mechanisms, and therefore, mutations in these genes can increase the risk for developing EBV-associated lymphomas: controlling T-cell receptor-stimulated $Mg2^+$ influx and concentrations (magnesium transporter 1, *MAGT1* gene) (22), modulating the SH2 domain-mediated interactions in signaling lymphocyte activation molecule (SLAM)-mediated activation (SH2 domain-containing 1A, *SH2D1A* gene) (23), sustaining the proliferation of activated lymphocytes by *de novo* mutations in genes associated with the pyrimidine synthesis pathway (nucleotide cytidine 5′ triphosphate synthases1, *CTPS1* gene) (24), activation of MAP-kinase cascade *via* guanine-nucleotide-exchange factors (RAS guanyl-releasing protein 1, *RASGRP1* gene) (25), and mediating critical signals from the T-cell receptor and activated lymphocyte-specific protein tyrosine kinase (interleukin-2-inducible T-cell kinase, *ITK* gene) (26). Although the mechanism of cancer immunosurveillance has been suggested in a minority of PAD with functional T cell defects, some EBV-associated cancer due to monogenic IEI can affect B cell terminal development and also present with antibody deficiency and lack of specific immunoglobulin production mimicking common variable immunodeficiency (CVID)-like phenotype (27, 28).

GENOME INSTABILITY AND MUTATION

Monogenic diseases of chromosome instability and DNA repair defects affecting steps of detection, removal, or further modification of the damaged DNA, and resynthesis and ligation of DNA strands can predispose both to immunodeficiency and cancer (29). T- B- receptor rearrangements [V(D)J recombinations] require the non-homologous end joining (NHEJ) pathway to process/repair double-strand DNA breaks and loss of function in various components of the NHEJ machinery present with T- B- severe combined immunodeficiency (SCID) (30, 31). In patients treated with hematopoietic stem cell transplantation, or carrying hypomorphic mutations in NHEJ factor encoding genes, survival may be associated with the development of hematological cancers, carcinomas, and sarcomas (5). Patients with DNA repair defects have a higher risk of EBV infections since the encoded viral proteins are implicated in the deregulation of DNA damage response signaling pathways (32). EBV infection disturbs ATM-mediated response (during the G2/M cell cycle *via* LMP1 and EBNA3C nuclear antigens) consistent with more frequent detection of EBV early antigen antibodies in patients with ataxia-telangiectasia in whom the incidence of lymphoma is increased (33, 34). Moreover, EBV attenuates DNA-dependent protein kinase and Artemis activities by depleting the p350/DNA-PK catalytic subunit and interacting with EBNA2, leading to a markedly increased incidence of EBV-induced lymphoproliferation in patients with pathogenic mutations in the *PRKDC* and *DCLRE1C* genes, up to 50% (34, 35).

Class switch recombination (CSR) and somatic hypermutation in peripheral B cells have a role in increasing the diversity of immunoglobulin classes as well as affinity maturation, which is accomplished by a large number of proteins involved in NHEJ, base excision repair, and mismatch repair (36). Ataxia-telangiectasia, Nijmegen breakage syndrome, Bloom's syndrome, and constitutional mismatch repair deficiency (CMMRD) syndromes are the main immunodeficiencies within this category and the patients usually develop lymphomas (5, 37). Activation-induced cytidine deaminase (AICDA) and uracil DNA glycosylase (UNG) deficiencies specifically affect the CSR in B cells, presenting as a PAD known as hyper IgM syndrome with an increased incidence of hematologic cancers (38, 39).

Dysregulations in epigenetic modifications and chromatin remodeling may result in genomic instability and syndromic features mainly in the immunological and neurological systems (40). Genes underlying immunodeficiency with centromeric instability and facial anomalies (ICF) syndrome are responsible for DNA methylation and critical epigenetic modification during lymphocyte development, chromatin structure remodeling, and physiological DNA breaks (41). ICF patients display DNA hypomethylation mainly affecting satellite 2 and 3 repeats of pericentromers, which is very common in cancer cells (42), in line with the higher incidence of cancers in these patients (43). Of note, cases with ICF syndrome due to hypomorphic mutations may

manifest without facial and neurologic symptoms, mimicking CVID-like phenotype with only antibody defects or recurrent infections and they may survive longer with a higher chance for the development of cancers (44, 45).

ENABLING REPLICATIVE IMMORTALITY

This cancer hallmark is described as an independently driven process involving the elongation of telomeres by reactivation of telomerase reverse transcriptase and increasing the cell proliferative capacity (46, 47). This process is regulated by the catalytic subunit of telomerase reverse transcriptase (TERT) that connects this hallmark to metabolic reprogramming, apoptosis, and tumor invasion (48). Thus, TERT and its associated elements could directly connect the various hallmarks of cancer (49). Dyskeratosis congenita (DKC) is a complex of syndromic features caused by defects in these proteins, which can result in a severe form of Hoyeraal–Hreidarsson syndrome due to short telomeres and genome instability (50–52). Recently, Coats-plus syndrome with mutations in *STN1* and *CTC1* have been described and linked to immunodeficiency with abnormal telomeres. This group of genetic abrogations frequently predisposes patients to myelodysplasia and leukemia (53, 54). Intriguingly, several cases of dyskeratosis congenita can show specifically with the initial presentation of antibody deficiency, and due to misclassification, they are more prone to the development of long-term complications like malignancies (27, 55).

TUMOR-PROMOTING INFLAMMATION

Although chronic inflammation occurs in most IEI patients with a delayed diagnosis and poor treatment, some subgroups of patients can develop unrestrained systemic inflammatory reactions despite immunomodulation, which may lead to cancer (56, 57). This cancer hallmark is well characterized in disturbance of immune regulation with colitis (due to a defective IL10-STAT1 pathway) (58, 59) and predisposition to mucocutaneous candidiasis (mainly due to a defective IL17 pathway) (60) that can increase the susceptibility to lymphoma and carcinoma, respectively.

Moreover, CVID, as the most common symptomatic form of antibody deficiency, also had a higher rate of chronic inflammation despite regular and appropriate treatment (61). Due to its high prevalence, the majority of IEI cancer patients have a clinical diagnosis of CVID (10). CVID is a heterogeneous disease, and there is an ongoing debate about criteria for diagnosis that mainly rely on the fulfillment of specific immunologic criteria. Therefore, CVID is considered as an umbrella term constituting several different humoral immune failures and antibody production impairment due to unknown monogenic, polygenic, or epigenetic defects (27, 28). However, the main suggested pathogeneses for the cancer phenomenon in CVID patients are immune dysregulation and chronic infection due to lack of mucosal immunity (absence of IgA in selected CVID patients). Therefore, subsequent inflammation might be a tumor-predisposing factor especially towards gastric cancers in CVID cases (62–65). The same phenomenon can be present in other entities of PAD with low IgA levels including congenital agammaglobulinemia and IgA deficiency (62, 66–68). Of note, a minority of CVID patients can present chromosomal radiosensitivity due to disruption of DNA repair machinery and must be considered for tumorigenesis due to genome instability and regular screening for cancer and avoidance of malignancy triggers must be added to their routine management (69, 70).

RESISTING CELL DEATH

Autoimmune lymphoproliferative syndrome (ALPS) is the porotype of IEI, which is associated with apoptosis defects and malignancies (71). The most well-established activity of affected proteins in the FAS–FAS ligand and Caspase pathway is to mediate the apoptotic death of either virus-infected cells or cancer cells when engaged by a cytotoxic lymphocyte (72). Although lymphoma has been reported as the most common type of malignancy seen in these patients, additional types of cancers in this population suggest a broader cancer predisposition as previously observed with somatic *FAS* mutations (73–76). Since apoptosis has an important role in the development, function and maintenance of the immune system, it controls the duration of immune responses to foreign antigens and deletion of auto-reactive T and B lymphocytes (77). Similarly, several abnormalities in T- and B-cell apoptosis in patients with humoral immunodeficiencies such as CVID have been reported and suggested to be underlying the higher rate of malignancy, particularly lymphoma, in this group of patients (78, 79).

SUSTAINING PROLIFERATIVE SIGNALING

Self-sufficiency in growth signals, bypassing various checkpoints, may be implicated in a vast number of patients with IEI and cancers (80). Immune system defects and dynamical compensation in physiological circuits lead to increased production of stimulatory factors mainly in patients with stem cell and myeloid developmental defects (81). Congenital neutropenias and other syndromic IEI (Wiskott-Aldrich, Shwachman-Diamond, MonoMac and immuno-osseous dysplasia syndromes) affecting early non-lymphoid stem cell lineages can manifest with myelodysplasia and leukemia (82).

Higher rates of diagnosis during recent years and detailed follow-up of autosomal dominant gain-of-function defects in signal transducer and activator of transcriptions (STAT) (83, 84), caspase recruitment domain family members (CARD) (85, 86) and NACHT, LRR, and PYD domain-containing proteins (NLRP) have shown an increased incidence of both hematological and solid tumors (87). Of note in the PAD category, several gain-of-function genetic defects in the signaling of phosphoinositide 3-kinase (PI3K) and nuclear factor κ-enhancer of activated B cells (NF-κB) have been shown to be involved in the dysregulation of the adaptive immune response and continuous lymphoid tissue growth, thus increasing the susceptibility to lymphoma (88–92). Of note, a minority of patients with NF-κB defects also presented avoiding cellular immune destruction mainly due to abrogated CD8 T-cell immunity (93, 94).

EVADING GROWTH SUPPRESSORS

The diverse functions of tumor suppressors vary from proliferation restriction to the regulation of regenerative processes in different human cell types (95). However, these elements modulate the proliferation and differentiation of immune cells to protect their genomic integrity during physiologic cellular metabolic and proliferative stress (96). The existence of multiple tumor suppressor family members (e.g., p53, retinoblastoma, and Hippo genes) may allow certain family members to have taken on specific roles in the enhancement of hematopoietic stem cell regeneration, DNA repair, chromosome remodeling, and cell-cycle checkpoint for selecting the desired modification (97).

One of the main tumor suppressor pathways conferring immunodeficiency and susceptibility to cancers is the posttranslational regulation of phosphatase and tensin homolog (PTEN) (98). PTEN is a negative regulator of PI3K signaling and is very commonly mutated in human cancers. Since PTEN is essential during early development, only heterozygous loss-of-function mutants have been reported in individuals with CVID-like phenotype with lymphoproliferation and hyperplasia (99). The prototypical tumor suppressor gene and pathway is p53, which is also a key pathway component affected in a majority of DNA repair defects associated with immunodeficiency and cancers (e.g., patients with *ATM* and *MRE11* mutations) (100).

Dedicator of cytokinesis 8 (DOCK8) can act as a tumor suppressor in non-hematopoietic tissues by directly affecting apoptosis through regulation of migration, morphology, adhesion, and growth of cells, apart from its probable role in $CD8^+$ T cells for tumor surveillance (101). Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is upregulated in activated naïve T cells through the T-cell receptor and subsequent engagement of the costimulatory receptor CD28 (102). This suppressive molecule acts as co-inhibitory and mutation in the autosomal dominant form impairs the function of regulatory T cells, thus increasing the risk for autoimmunity, chronic inflammation, and cancers (103). Patients with lipopolysaccharide-responsive and beige-like anchor protein (LRBA) deficiency, a crucial molecule for recycling of CTLA4 and the function of regulatory T cells, present a similar CVID-like phenotype with the development of both hematological and solid tumors (104, 105).

DEREGULATING CELLULAR ENERGETICS

IEIs associated with sustaining proliferative signaling induce endoplasmic reticulum stress, unfolded protein response, destabilization of mitochondrial membrane potentials, and disturbed energy metabolism (106, 107). Recent findings also suggest that there may be a common pathogenic mechanism that connects a high prevalence of cancer, metabolic disorders, atherosclerotic cardiovascular disease, and insulin-resistant diabetes in carriers of some DNA repair defects, in particular *ATM* mutations (108). Mutations of genes related to NHEJ and IEI disorders associated with chronic inflammation result in age-associated pathological conditions due to their roles in metabolic regulation in response to DNA damage avoiding further genomic instability (109, 110).

These defects in DNA repair and uncontrolled inflammation may induce stem cell exhaustion, cellular senescence, immunosenescence, low-grade chronic inflammation, activation of PI3K signaling, defective autophagy, and mitochondrial genome instability. It has been shown that ATM-dependent stress and dysregulation of inflammatory pathways mediate predisposition to both the metabolic syndrome and cancer (111).

INDUCING ANGIOGENESIS

A series of well-orchestrated cellular adaptations occur to stimulate angiogenesis and enhance the survival of tumors in hypoxic conditions (112). Gain-of-function somatic mutations in RAS-associated genes (*KRAS* and *NRAS*) can result in RAS-associated autoimmune leukoproliferative disease (RALD) with lymphocytosis and lymphoproliferation, a phenocopy of ALPS (113, 114). The affected proteins are GTPases that serve as a signaling switch molecule, coupling receptor activation by specific growth factors with downstream effector pathways. After cancer-related hypoxia responses, in patients with RALD, the production of vascular endothelial growth factor (VEGF) is enhanced (115). Therefore, the over-activation of RAS signaling significantly stimulates angiogenesis and blocks apoptosis in hypoxic conditions (116).

Furthermore, in cancers associated with defective innate or adaptive immune responses, the balance between pro- and anti-angiogenic factors is perturbed by dysregulated cytokine production by innate immune cells (117). Increased inflammatory mediators as a consequence of antibody deficiency, diseases of immune dysregulation, and autoinflammatory diseases contribute to neoplasia by stimulation of angiogenesis, where a change confers a survival advantage to a tumor cell (56, 118). Therefore, the promotion of angiogenesis in the IEI tumors accelerates the migration of endothelial cells and formation of new blood vessels, and distorted and enlarged vascular architecture with increased permeability and irregular blood flow (119).

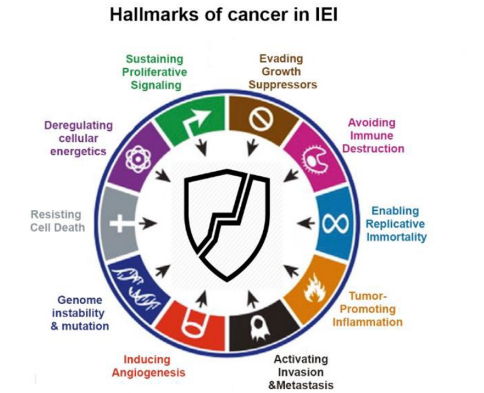
ACTIVATING INVASION AND METASTASIS

A selected group of IEIs faces aggressive oncogenic risks due to an increased susceptibility for viral replication and persistence (120). Among those, transforming viral infections with a distant invasion have been reported by human papillomavirus (HPV infection in Epidermodysplasia verruciformis and WHIM syndrome) (121, 122) and herpes viruses family (particularly EBV susceptibility in immune dysregulation diseases). Of note, both groups of patients with HPV (mainly WHIM syndrome) and EBV infection susceptibility can mimic the phenotype of CVID-like due to their predominance of humoral immunodeficiency. Although both HPV and EBV oncoviruses have undertaken different powerful anti-apoptotic and proliferative programs, they can directly induce metastasis in infected tumor cells. In HPV-associated IEIs, E6 and E7 proteins can contribute to tumor invasion by impacting epithelial-to-mesenchymal transition (123, 124), while in EBV infection, the LMP2A protein can promote differentiation,

survival, and cell growth by activating the PI3K pathway and pathways mediating cell mobility and invasion (125).

FUTURE DIRECTIONS AND CONCLUDING REMARKS

The evaluation of the hallmarks of cancer in IEI patients helps to explain the multistep nature of oncogenesis in different forms of immune defects/dysfunction (**Figure 1**). This outlines the complexity of the development of cancer in each entity of IEIs, requiring the progressive acquisition of different necessary cellular hallmarks that constitute a malignant phenotype. The distribution of distinct types of cancers in patients with specific genetic defects highlights the cell-specific predisposition to an intrinsic cause or extrinsic exposure in the context of the genetic background of the host and the selective pressures imposed by the tissue microenvironment. The analysis of a cancer hallmark model would also facilitate understanding about the process of IEI carcinogenesis to relevant treatment. Recently, cancer hallmarks have been reorganized into seven updated compact parameters (126). It has been suggested to consider altered stress



International Union of Immunological Societies categories	Disease	Genes	Cancer hallmarks
Immunodeficiencies affecting cellular and humoral immunity	T-B- SCID	*RAG1* *RAG2* *DCLRE1C* *PRKDC* *NHEJ1* *LIG4* *AK2*	
	Combined immunodeficiency with EBV infection	*CORO1A* *RHOH* *STK4* *TNFRSF4* *ITK*	
	MHC class I deficiency	*TAP1* *TAP2* *TAPBP* *B2M*	
	DOCK8 deficiency	*DOCK8*	
Combined immunodeficiencies with associated or syndromic features	Syndromic DNA repair defects	*ATM* *NBS1* *MRE11* *BLM* *POLE1* *POLE2* *POLD1* *POLD2* *LIG1 RNF168*	
	Coats plus syndrome	*STN1* *CTC1*	
	NK cell deficiency with EBV infection	*MCM4* *FCGR3A*	
	Immunodeficiency with centromeric instability and facial anomalies	*DNMT3B* *ZBTB24* *CDCA7* *HELLS*	
	Wiskott-Aldrich syndrome	*WAS*	
	Constitutional mismatch repair deficiency	*PMS2*	
	Thymic defects	*22q11.2* *TBX1* *FOXN1*	
	Immuno-osseous dysplasias	*RMRP* *MYSM1*	
	Hyper IgE syndromes	*STAT3* *CARD11*	
Predominantly antibody deficiencies	Common variable immune deficiency	*Not known*	
	Good syndrome	*Not known*	
	IgA deficiency	*Not known*	
	Hypogammaglobulinemia	*CD19* *CD81* *MS4A1* *CR2* *TNFRSF13B* *TNFRSF13C* *TNFSF12* *MOGS* *IKZF1* *IRF2BP2*	
	Hyper IgM syndrome	*AICDA* *UNG* *INO80* *MSH6*	
	Activated PI3K-delta syndrome	*PIK3CD* *PIK3R1*	
	NFKB Deficiency	*NFKB1* *NFKB2* *CARD11*	
	PTEN Deficiency	*PTEN*	

International Union of Immunological Societies categories	Disease	Genes	Cancer hallmarks
Bone marrow failure	Dyskeratosis congenita	*DKC1* *NHP2* *NOP10* *RTEL1* *TERC* *TERT* *TINF2* *TPP1* *DCLRE1B* *PARN* *WRAP53* *ACD* *NOLA2* *NOLA3* *SRP72* *SAMD9* *SAMD9L* *TP53*	
	Fanconi anemia	*FANCA* *FANCB* *FANCC* *FANCD2* *FANCE* *FANCF* *FANCI* *FANCL* *FANCM* *PALB2* *SLX4* *ERCC4* *UBE2T* *MAD2L2* *RFWD3* *XRCC2* *XRCC9* *BRCA1* *BRCA2* *RAD51* *RAD51C*	
Diseases of immune dysregulation	Familial hemophagocytic lymphohistiocytosis	*PRF1* *UNC13D* *STX11* *STXBP2* *FAAP24*	
	Regulatory T cell defects	*FOXP3* *CTLA4* *LRBA* *STAT3*	
	Autoimmunity with or without Lymphoproliferation	*AIRE* *ITCH* *ZAP70* *TPP2* *JAK1* *PEPD*	
	Autoimmune lymphoproliferative syndrome	*TNFRSF6* *FASLG* *CASP10* *CASP8* *FADD*	
	Immune dysregulation with colitis	*IL10RA* *IL10RB* *NFAT5*	
	Susceptibility to EBV and lymphoproliferative conditions	*SH2D1A* *CD27* *CD70* *CTPS1* *RASGRP1* *RLTPR/CARMIL2* *MAGT1* *PRKCD* *TET2*	
Congenital defects of phagocyte number or function	Congenital neutropenias	*ELANE* *GFI1* *HAX1* *VPS45* *SMARCD2*	
	MonoMac syndrome	*GATA2*	
Defects in intrinsic and innate immunity	Mendelian susceptibility to mycobacterial disease	*RORC* *JAK1*	
	Epidermodysplasia verruciformis	*TMC6* *TMC8* *CXCR4* *CIB1*	
	Predisposition to mucocutaneous candidiasis	*IL17RA* *IL17RC* *IL17F* *STAT1* *TRAF3IP2*	
Autoinflammatory disorders	Defects affecting the inflammasome	*NLRP3* *NLRP12* *NLRC4* *NLRP1* *HAVCR2*	
Phenocopies of inborn errors of immunity		*TNFRSF6* *KRAS* *NRAS*	

FIGURE 1 | Cancer hallmark activation in different types of monogenic inborn errors of immunity (IEI) according to the International Union of Immunological Societies classification (1, 3).

TABLE 1 | Therapeutic and preventive approaches successfully used or potentially can be implemented to prevent primary immunodeficiency-associated cancer hallmarks.

Hallmark or Process	Agent or Vause	Drug or Modality
Avoiding immune destruction	EBV infection**	EBV-specific cytotoxic T lymphocytes
	Costimulatory agonist	Anti-GITR, anti-ICOS, anti-OX40, and anti-CD27
	Regulatory T cells**	Anti-CD25
Deregulating cellular energetics	Immunometabolism	IDO1 inhibitors, A2AR antagonists, Arginase inhibitors, and Glutaminase inhibitors
Evading growth suppressors	Dual checkpoint blockade*	Anti-CTLA-4 (Ipilimumab), anti-PD1 (Nivolumab), anti-PDL1 (Atezolizumab), anti-TIM3, anti-LAG3, anti-TIGIT, and anti-VISTA
Genome instability and mutation	DNA repair defect*	Decrease radiation exposure
	Epigenetic changes*	DNMT inhibitors and HDAC inhibitors
Inducing angiogenesis	RAS-associated autoimmune leuko-proliferative disease	Cetuximab, Pantitumumab, and Bevacizumab
Sustaining proliferative signaling	EBV infection**	Butyrate and Ganciclovir
	HPV infection*	L1 virus-like particles vaccine
	BTK activation*	Ibrutinib and Acalabrutinib
	PI3K activation**	Rifampicin, Buparlisib, Alpelisib, Nemiralisib, and Idelalisib
	PI3K or NFKB activation**	Rituximab, Ibritumomab Tiuxetan, and Tositumomab
	mTOR activation**	Everolimus
	MAPK/ERK activation**	Trametinib
	Stem cell and myeloid development defects	Bone marrow transplantation, CSF1R inhibitor, and HDAC inhibitors class IIa
	Cytokines	JAK inhibitors, TGF inhibitors, and MET inhibitors
Tumor-promoting inflammation	*H. pylori* infection*	Standard triple therapy consisting of proton pump inhibitor, clarithromycin, and amoxicillin
	Chronic inflammation*	Nonsteroidal anti-inflammatory drugs

EBV, Epstein–Barr virus; GITR, glucocorticoid-induced TNFR-related protein; ICOS, Inducible T-cell COStimulator; IDO1, Indoleamine 2;3-dioxygenase 1; A2AR, Adenosine 2A receptor; CTLA4, Cytotoxic T-lymphocyte protein 4 precursors; TIM3, T-cell immunoglobulin and mucin domain 3; LAG3, Lymphocyte-activation protein 3; TIGIT, T-cell Immunoreceptor With Ig And ITIM Domains; VISTA, V-domain Ig suppressor of T-cell activation; DNMT, DNA Methyltransferase; HDAC, Histone deacetylase; HPV, human papillomavirus; PI3K, Phosphoinositide 3-kinase; CSF1R, Colony-stimulating factor 1 receptor; NFKB, nuclear factor kappa B; JAK, Janus kinase; TGF, Transforming growth factor.

***Genes/pathways very important in the pathogenesis of antibody deficiencies.*

**Genes/pathways important in the pathogenesis of antibody deficiencies.*

response favoring overall survival by combining defects of genome instability and mutation, enabling replicative immortality, tumor-promoting inflammation, and resisting cell death hallmarks (126). Moreover, a new hallmark for abetting microenvironment has been offered to cover cancer etiologies related to communication between the dynamic microenvironment of the affected organ and stromal cells (5, 126). IEI genes underlying each hallmark might help to investigate whether these newly proposed revisions are functionally and molecularly relevant.

Based on several lines of evidence, PAD patients constitute the highest proportion of IEI cases affected by malignancies. Moreover, several monogenic defects with different involved cancer hallmarks can mimic the clinical and immunologic phenotypes of PAD patients, mainly CVID. The abovementioned overview about IEI-induced and PAD-induced cancers indicated that these malignancies are amenable to immune prophylaxis by vaccines, prophylactic radiation limitation, and, most recently, targeted therapy. However, future clinical efforts in preventing or treating gene-specific-associated malignancies represent a combination of antiviral therapies, agents that induce cytotoxicity events, agents that improve DNA repair machinery, and agents that are used to successfully treat cancers with antagonists and agonists for IEI tumor stimulators and repressors. **Table 1** illustrates some of the most promising approaches that could be clinically tested in both PAD and IEI patients. Of note, other monogenic IEIs mainly with combined immunodeficiency and immune dysregulation also have diverse cancer hallmarks as PAD patients; however, they are more likely to be transplanted due to the risk of cancer, whereas most PADs may not be transplanted. The treatment of cancers in the context of immune defects, however, remains challenging and a detailed molecular investigation and multi-omics analysis of both germline and somatic (tumor) genome may increase the number of potential therapeutic targets and also further provide clues of potential resistance to therapy.

AUTHOR CONTRIBUTIONS

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Serum Protein Electrophoresis May Be Used as a Screening Tool for Antibody Deficiency in Children and Adolescents

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Background: Patients with antibody deficiency may experience exceptionally long diagnostic delays, increasing the risk of life-threatening infections, end-organ damage, mortality, and health costs.

Objective: This study aimed to analyze serum protein electrophoresis and verify the correlation between calculated globulin (CG, total protein minus albumin levels) or electrophoretically determined serum gamma globulin fraction (Gamma) with IgG levels in children and adolescents under 18 years old (yo).

Methods: We analyzed serum protein electrophoresis (GC or Gamma) and IgG levels from 1215 children and adolescents under 18 yo, classified into 5 age groups. We verified the correlation between CG or Gamma with serum IgG levels.

Results: Serum IgG levels varied according to age groups (from 4.3 ± 2.3 g/l in children under 6 months old to 11.4 ± 3.2 g/l in adolescents in the 10-<18 yo group). CG sensitivity and specificity to detect IgG below the reference range for all patients were 93.1% and 81.8%, respectively, and varied according to age group. Gamma sensitivity and specificity for all patients were 100% and 87.8%, respectively, and varied according to age group as well. We found serum IgG levels below the age reference level in 29 patients (2.4% of the cases) using CG or Gamma levels.

Conclusion: Both CG and Gamma levels may be of utility as a screening tool for earlier diagnosis of antibody deficiency in children and adolescents under 18 yo.

Keywords: antibody deficiency, calculated globulin (CG), gamma globulin fraction, children, immunoglobulin G (IgG), serum protein electrophoresis (SEP)

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INTRODUCTION

Antibody deficiencies are the most commonly reported immunodeficiencies worldwide and may be either primary or secondary. Primary antibody deficiency (PAD) refers to a heterogeneous group of genetic disorders characterized by an intrinsic impairment in antibody production or function (1).

Inborn errors of immunity (also known as Primary Immune Deficiencies – PIDs) are a group of more than 400 diseases caused by monogenic germline mutations and characterized by increased susceptibility to infectious diseases, autoimmunity, autoinflammation, allergy, and malignancy (2). While on a global scale the commonest causes of secondary immunodeficiency include HIV and malnutrition, primary antibody deficiencies make up by far the largest subset of inborn errors of immunity including both (3) predominantly antibody deficiencies or in categories associated with defects in innate immune cells or T cells (4). Taken together, antibody deficiencies are present in 70-80% of all PIDs (5) and are recognized to be both under-diagnosed and under-reported in a systematic review of PID registries (6).

The diagnosis of quantitative antibody deficiency is generally straightforward using serum immunoglobulin measurement (7). However, patients frequently experience long delays before diagnosis and treatment (8–10). This diagnostic delay is often measured in years and can lead to end-organ damage (11) and decreased survival (12); while prompt and appropriate treatment decreases morbidity and mortality [reviewed by Perez et al. (13)]. Early diagnosis thus reduces health care expenses and leads to better health outcomes for patients with PIDs (14).

Screening methods that improve earlier identification of antibody deficiencies are of key importance in reducing diagnostic delay. T cell receptor excision circle (TREC) (15) or κ (kappa)-deleting excision circle (KREC) (16) methods are available for newborn screening of severe forms of PIDs but are not yet widely offered (17). While very successful in the detection of severe combined immunodeficiency (SCID) and potentially a small subset of agammaglobulinemia without B cells, these tests do not effectively detect diseases with a normal number of T and B cells and those with later onset, such as common variable immunodeficiency (CVID) (18).

A number of studies have demonstrated that calculated globulin (CG) can be used as a low-cost screening method for antibody deficiencies in adults (19, 20). CG is derived from the difference between total protein and albumin levels and can be calculated automatically, often as part of liver function tests (LFTs).

This study is the first to establish a correlation between CG, electrophoretically determined gamma globulin fraction (Gamma) and IgG levels in children and adolescents by age range in a Brazilian population sample. Unlike previous publications, we used protein electrophoresis to determine CG and Gamma, allowing us to correlate those with IgG levels in the same groups. Both yielded significant correlations with the IgG levels, showing that CG or Gamma could be used to screen for antibody deficiencies in children and adolescents.

MATERIALS AND METHODS

Participant Details

In line with the Brazilian Ministry of Health and the Helsinki Convention's rules and regulations participants aged from 0 to 18 years were recruited with consent from three different Allergy/Immunology clinics in São Paulo State, Brazil. Inclusion criteria were outpatients aged less than 18 years old, with clinically stable conditions, and informed consent. Exclusion criteria were age above 18 years old, unstable clinical conditions, and lack of informed consent.

All patients were referred for possible immunologic or allergic conditions. One hundred and eighty-eight had a final diagnosis of PID (8.9% of the cases) and 29 presented with antibody deficiency (2.4% of the cases). We did not include any patients with secondary immunodeficiency.

A 5mL blood sample was collected and patients were able to choose which laboratory undertook the analyses. All laboratories were accredited according to the Associação Brasileira de Normas Técnicas (ABNT NBR ISO 15189) (21), the Brazilian Society of Clinical Pathology (PALC) (22), and were contacted to determine equipment and testing methodology.

Laboratory Measurements

IgG, IgA, IgM values were determined by immunoturbidimetry (Roche COBAS 6000, Roche Diagnostics International Ltd, CH-6343, Rotkreuz, Switzerland). IgG reference values were based on Adeli et al. (23). Serum protein electrophoresis (SEP) was performed using Hydrasys (Sebia, Paris, France) instruments and Hydragel Protein (E) gels (Sebia, Paris, France). The visualization of the gel provided qualitative analysis, while reading of the agarose gels on a Sebia reader provided protein profiles for relative quantitative analysis by Hydrasys 2 Scan (Sebia, Paris, France) scanning system. CG values were obtained by subtracting the albumin levels from total protein values. The gamma globulin fraction was directly determined by protein electrophoresis.

Statistical Analysis

One thousand three-hundred thirty five (1335) consecutive patients from ages 0 to 18yo were recruited. **Figure 1** depicts the flow of excluded samples.

The Kruskal-Wallis test followed by the Mann-Whitney U test was used for IgG, CG, and Gamma levels in both studies. The Bonferroni method was used to adjust p values for multiple variables. The assumptions of normality of data distribution and homogeneity of variances were checked by the Shapiro Wilk Test and Levene Test. The chi-square test was applied to compare the frequency of occurrence between males and females in each age group. Linear regressions were performed to explore the association between IgG *vs.* CG and IgG *vs.* Gamma globulin fraction models. One-way ANOVA followed by a *post hoc* Bonferroni's test was used to compare age groups.

The accuracy of the obtained discriminant value was interpreted based on the AUC and classified as: "perfect" (AUC = 1), "exceptional" (0.9 ≤ AUC <1), "excellent" (0.8 ≤ AUC <0.9), "acceptable" (0.7 ≤ AUC <0.8) and "poor" (AUC <0.7), noting that the AUC is not statistically different from that obtained at random for AUC values ≤ 0.5 (24). The Youden index was calculated to confirm the discriminant

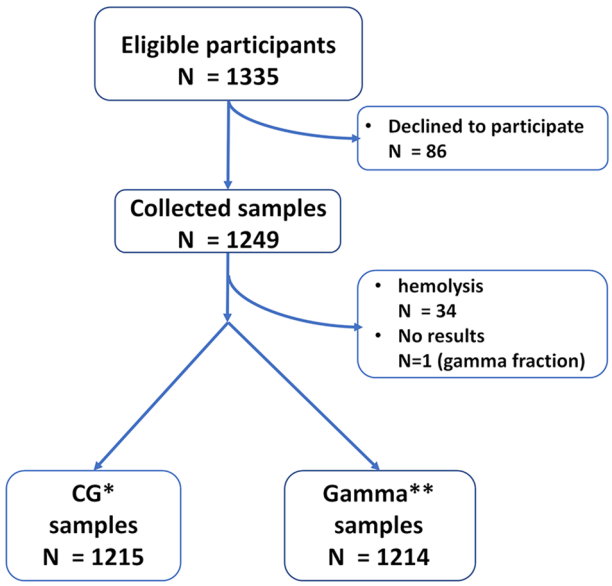

FIGURE 1 | Sample flow. *CG – Calculated Globulin. **Gamma – Gamma Globulin fraction.

score, defined as the highest value observed for the following operation: sensitivity + specificity – 1 (25).

Receiver operating characteristic curves were created to identify discriminating CG and Gamma globulin cutoff values.

All analyses were conducted in PASW statistics 18.0 software (SPSS Inc., Chicago, USA), adopting a significance level (α) of 5% ($P < 0.05$).

RESULTS

The study included 1249 patients. CG analyses included 1215 samples while Gamma analyses included 1214 samples. See **Figure 1** for recruitment and sample flow details.

Correlation Between IgG and CG Values

Descriptive data for the IgG x CG analysis are shown in **Table 1**. There was a stepwise increase observed for both IgG and CG with age.

In analyzing discriminant cutoff values between patients with levels below the reference and normal for IgG from CG, the predictive power was classified as excellent to exceptional (AUC from 0.91 to 0.96). AUC was significant and with acceptable accuracy for all age groups, except those younger than 1 yo (**Table 2**). For these groups, we could not establish discriminant CG cutoff values between patients with levels below the reference and normal for IgG because there were no patients with hypogammaglobulinemia. Sensitivity values ranged from 90.9% to 100.0% in the remaining age groups. The specificity values ranged from 80.2% to 94.7%. Good accuracy was also observed for the cutoff value obtained regardless of the participants' ages (AUC = 0.916, P <0.001, sensitivity = 93.1% and specificity = 81.8%).

A significant positive relationship in simple linear regression was observed between GC and IgG values for all age groups analyzed separately or in a combined analysis. CG values were able to significantly explain part of the IgG values variance for all age groups: 1 to 5 mos., 67%, (**Figure 2A**); 6 to 11mos., 46%M, (**Figure 2B**); 1 to 3 years, 63%, (**Figure 2C**); 4 to 9 years, 65%, (**Figure 2D**); 10 to <18 years, 68%, (**Figure 2E**); Additionally, when analyzing the entire cohort, CG values explained 68% of IgG % (**Figure 2F**).

TABLE 1 | Characteristics of patients studied for the IgG *vs* CG correlation[a].

Age Group	Age (years)	IgG (g/L)	CG (g/L)	% males
1 to 5 mos (n = 23)	0,3 ± 0,1	4,3 ± 2,3	21,1 ± 4,2	52,2
6 to 11 mos (n = 56)	0,7 ± 0,1	5,4 ± 2	23 ± 4,2	42,9
1 yo to <4 yo (n = 364)	1,8 ± 0,8	8,4 ± 3	26,8 ± 4,3	53,8
4 yoto <10 yo (n = 442)	6,3 ± 1,7	10,2 ± 3	27,9 ± 4	51,7
10 yo to <18 yo (n = 330)	13 ± 2,3	11,4 ± 3,2	29,4 ± 4,5	55,2
All (n = 1215)	6,4 ± 4,8	9,7 ± 3,4	27,6 ± 4,6	52,9

[a]*Data are presented as mean ± SD.*

TABLE 2 | CG values as a function of IgG levels.

Age Group	AUC	95% CI	p value	CG Cutoff value (g/L)[a]	Sensitivity	Specificity	Number of patients with IgG	
							Below reference values	Normal
1 to 5 mos (n = 23)	–	–	–	–	–	–	0	23
6 to 11 mos (n = 56)	–	–	–	–	–	–	0	56
1 yo to <4 yo (n = 364)	0,965	0,92 - 1	<0,001	23,1	1	0,838	6	358
4 yoto <10 yo (n = 442)	0,951	0,91 - 0,99	<0,001	24,8	1	0,802	12	430
10 yo to <18 yo (n = 330)	0,945	0,85 - 1	<0,001	24,1	0,909	0,947	11	319
All (n = 1215)	0,916	0,87 - 0,96	<0,001	24,1	0,931	0,818	29	1186

AUC, area under the curve. CI 95%, 95% confidence interval.
[a]*CG values below which IgG levels were considered below reference.*

Correlation Between IgG and Electrophoretically Determined Gamma Globulin Fraction (Gamma) Fraction Values

Descriptive data for the Gamma globulin fraction analysis are shown in **Table 3**. A significant positive association was observed between the Gamma and IgG values (**Figure 3**) for all age groups, separately and for the combined analysis. Gamma values were able to significantly explain part of the variance in IgG values in all groups: 0 to 5 months (88% **Figure 3A**), 6 to 11 months (88%, **Figure 3B**), 1 to 3 years old (91%, **Figure 3C**), 4 to 9 years old (92%, **Figure 3D**), 10 to < 18 years old (92%, **Figure 3E**). For the combined analysis of all samples, Gamma values explained 93% of the IgG values variance (**Figure 3F**).

In analyzing discriminant Gamma cutoff values between patients with levels below the reference level for IgG, the predictive power was classified as exceptional (AUC from 0.963 to 1.00), with AUC being significant and acceptable accuracy for all age groups, except those younger than 1 yo (**Table 4**). For these groups, we could not establish discriminant Gamma cutoff values between patients with levels below the reference and normal for IgG because there were no patients with hypogammaglobulinemia.

The sensitivity values were 100% for all groups, and specificity varied between 97.9% and 99.7% in all age groups. Exceptional accuracy was also observed for the cutoff value obtained for the combined age groups (AUC = 0.963, P <0.001, sensitivity = 100%, and specificity = 87.8%, **Table 4**).

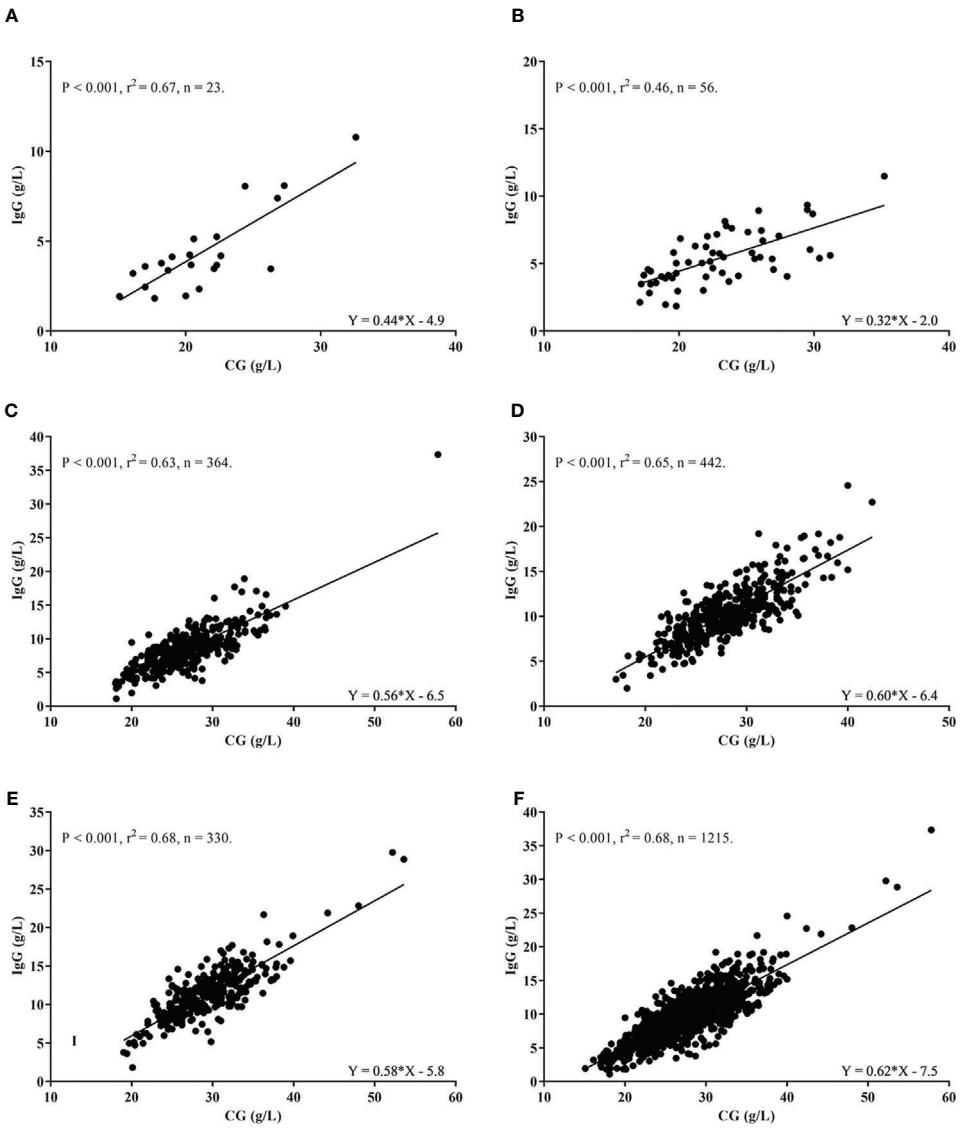

FIGURE 2 | Correlation between IgG (g/L) and Calculated Globulin values (g/L) according to age groups. **(A)** 1 to 5 months. **(B)** 6 to 11 months. **(C)** 1 to 3 years. **(D)** 4 to 9 years. **(E)** 10 to <18 years. **(F)** All age groups combined.

TABLE 3 | Characteristics of patients studied for the IgG *vs* Gamma fraction correlation[a].

Age Group	Age (years)	IgG (g/L)	Gamma (g/L)	% males
1 to 5 mos (n = 23)	0,3 ± 0,1	4,4 ± 2,4	4,2 ± 2,7	47,6
6 to 11 mos (n = 56)	0,7 ± 0,1	5,4 ± 2	5,5 ± 1,9	45,3
1 yo to <4 yo (n = 364)	1,8 ± 0,8	8,4 ± 2,7	8,3 ± 2,5	54,1
4 yoto <10 yo (n = 442)	6,3 ± 1,7	10,1 ± 2,9	9,9 ± 2,9	51,6
10 yo to <18 yo (n = 330)	13 ± 2,3	11,3 ± 2,9	11,1 ± 3	55,6
All (n = 1215)	6,4 ± 4,78	9,6 ± 3,2	9,4 ± 3,2	53,2

[a]*Data are presented as mean ± SD.*

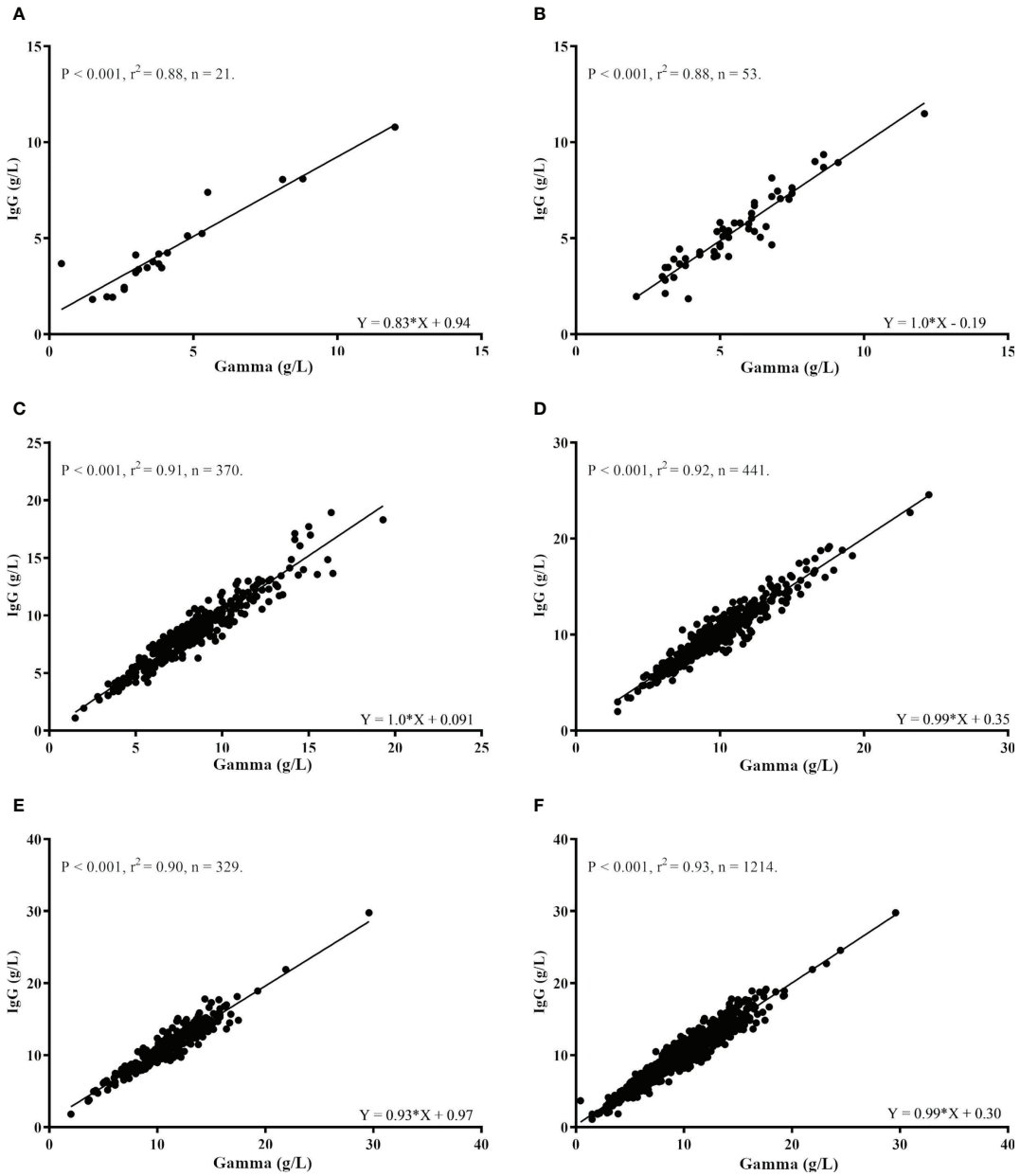

FIGURE 3 | Correlation between IgG (g/L) and Gamma globulin values (g/L) according to age groups. **(A)** 1 to 5 months. **(B)** 6 to 11 months. **(C)** 1 to 3 years. **(D)** 4 to 9 years. **(E)** 10 to <18 years. **(F)** All age groups combined.

TABLE 4 | Gamma fraction values as a function of IgG levels.

Age Group	AUC	95% CI	p value	Gamma Cutoff value (g/L)[a]	Sensitivity	Specificity	Number of patients with IgG	
							Below reference values	Normal
1 to 5 mos (n = 21)	–	–	–	–	–	–	0	21
6 to 11 mos (n = 53)	–	–	–	–	–	–	0	53
1 yo to <4 yo (n = 370)	1	1 - 1	<0,001	3,55	1	0,997	6	364
4 yoto <10 yo (n = 441)	0,997	0,99 - 1	<0,001	5,65	1	0,979	12	429
10 yo to <18 yo (n = 329)	0,995	0,99 - 1	<0,001	6,2	1	0,981	11	318
All (n = 1214)	0,963	0,95 - 0,98	<0,001	6,15	1	0,878	29	1185

AUC, area under the curve. CI 95%, 95% confidence interval.

[a]*Gamma values below which IgG levels were considered below reference.*

DISCUSSION

Primary and secondary antibody deficiencies are treatable conditions, frequently associated with diagnostic delays (8–10), leading to higher morbidity, mortality (13, 26), and overall costs of treatment (14).

This work shows that both CG and Gamma fraction can serve as correlates of IgG levels and could be used as screening methods for detecting antibody deficiency in children and adolescents. We found different cutoff values by age group, both for CG and Gamma, in keeping with the age dependent lower limit of the reference ranges for IgG (23). We demonstrate that CG or Gamma have a good to excellent correlation with IgG levels, independent of age group.

In previous studies, Jolles et al. (19) described CG as a screening method for adults in Wales, using the Architect Biuret method for total protein calculation and the bromocresol green method for albumin. The authors chose a cutoff value of CG < 18 g/L, which corresponded to a sensitivity of 0.82 and a specificity of 0.71 for an IgG < 3 g/l. Thereafter, Holding et al. (27) showed the results of an extensive screening program in England, using a rate biuret method or total protein and bromocresol purple for albumin. It is unclear if there were children or adolescents in the sample, but the authors chose a cutoff value for CG <18g/L, with a positive predictive value of 8.6% (7–11%) for IgG <3g/L. Pecoraro et al. (20), using the same methods as Jolles et al., chose a cutoff value of 19g/l to detect IgG levels below 6g/L, with a sensitivity of 70% and a specificity of 75%. This study was performed in adult Italian patients (>18 yo).

Assessment of the pediatric population and a different method for calculating total protein and albumin, namely serum protein electrophoresis, distinguish our study from those described above. In this regard, CG cutoff values were established for different age groups, ranging from 23.1 g/L in the 1 to 3 yo group to 24.8 g/L in the 4 to 9 yo group (see **Table 2** for details). This method's accuracy also varied among the age groups, with sensitivity ranging from 90.9% in the 10 to <18 yo group to 100% in the 1 to 3 yo and 4 to 9 yo groups. Specificity also demonstrated a variation from 80.2% in the 4 to 9 yo group to 94.7% in the 10 to <18 yo group.

Gamma globulin fraction cutoff values to discriminate individuals with low IgG levels varied depending on the age groups (see **Table 4**). Interestingly, both the sensitivity and the specificity of this method for the whole group (100% and 87.8%, respectively) was slightly higher than those of CG (93.15% and 81.8%, respectively). However, the number of individuals identified below reference levels for IgG in the total sample was the same (29 individuals).

For children under 1-year-old, we evaluated the correlation between IgG *versus* CG in two groups, according to the age in months. Although the numbers of individuals were smaller compared to the whole group, all groups under one year had significant correlations between the parameters. Diagnosis of a primary antibody deficiency is less frequent in this population, as immunoglobulin levels in the newborn relate to the maternal-fetal transfer of antibodies. The maternal-fetal transfer of immunoglobulins is dependent on several factors, including maternal levels of total IgG and specific antibodies, gestational age, placental integrity, IgG subclass, and nature of antigen (28). The nadir for IgG levels occurs at three months of age, but transient hypogammaglobulinemia can persist because of a prolonged nadir (29). These factors make the diagnosis of hypogammaglobulinemia in infants <1 yo challenging. Furthermore, the small number of patients younger than 1 year in our study limited our ability to reach definitive conclusions.

IgG makes up around 75% of total serum immunoglobulins, with IgA levels usually 4 to 5 times, and IgM levels 7 to 10 times lower than IgG (30). Therefore, both the sensitivity and specificity of the test to detect IgA, IgM and IgG subclass deficiency is expected to be much lower. Specific antibody deficiencies cannot be detected using CG or Gamma fraction screening methods.

The aim of these tests is to screen for antibody deficiencies, in particular IgG as the major immunoglobulin class in blood, however, subsequent definitive diagnosis will require follow on tests, such as measurement of quantitative immunoglobulin levels, followed by B and T cell studies, functional antibody testing and/or genomic tests as appropriate.

Calculated globulin or Gamma fraction as screening tools for detecting IgG antibody deficiency fulfills all of the rules proposed by Wilson and Jungner (31) and most of the revised rules proposed by Dobrow et al. (32). The tests are low cost, readily available, and regularly performed to diagnose or follow-up other diseases or as routine/baseline testing. Our study indeed shows that CG or Gamma fraction were able to detect 29 cases of abnormal low IgG levels, 2.4% of the cases.

One limitation of our proof of principle study is the nature of the sample population (enriched for patients who sought

Allergy/Immunology clinics and frequently presenting with a history of recurrent infections), which may lead to different levels of accuracy compared to other populations and the chosen cutoff values (23) may differ across settings. Another limitation (potentially an advantage), was the free patient choice of laboratories. This may impact the results, but is closer to real-life and clinical practice.

In conclusion, CG and Gamma fraction are simple screening methods for primary antibody deficiencies in children and adolescents. While this study did not include patients with secondary antibody deficiencies, CG screening detected secondary antibody deficiency in other studies (19). We have established age-dependent cutoff values for pediatric and adolescent patients using CG and Gamma fraction with the potential to decrease diagnostic delay, morbidity, mortality, and costs. In the future, it will be possible to introduce automated comments to prompt further investigation, such as IgG, IgM, and IgA determinations, when CG or Gamma fraction fall below the cutoff values, allowing earlier diagnosis and better outcome of antibody deficiency conditions. Further studies are needed in more general settings to evaluate the accuracy of these tests in a wider population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The University of São Paulo and the Federal University of São Paulo Ethics Committees' (approval number 3.340.392 and 3.499.511, respectively). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CP, data collection and manuscript writing. CA, patient selection and manuscript review. DS, patient selection and manuscript review. AC-N, study design, manuscript writing, and review. SJ study design and manuscript review. All authors contributed to the article and approved the submitted version.

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SJ has participated in advisory boards, trials, conferences, projects, and has been a speaker with CSL Behring, Takeda, Swedish Orphan Biovitrum, Biotest, Binding Site, Grifols, BPL, Octapharma, LFB, Pharming, GSK, Weatherden, Zarodex, Sanofi, and UCB Pharma.

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Diagnostic Vaccination in Clinical Practice

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Testing the antibody response to vaccination (diagnostic vaccination) is crucial in the clinical evaluation of primary immunodeficiency diseases. Guidelines from the American Academy of Allergy, Asthma & Immunology (AAAAI) provide detailed recommendations for diagnostic vaccination with pure pneumococcal polysaccharide vaccines (PPV). However, the degree of compliance with these guidelines and the utility of the guidelines in actual practice are undescribed. To address this, we systematically evaluated diagnostic vaccination in adult patients with suspected primary immunodeficiency diseases in a single tertiary center from 2011 to 2016 (n = 229). We found that full compliance with the AAAAI guidelines was achieved for only 39 patients (17%), suggesting that the guidelines are not easy to follow. Worse, interpretation according to the guidelines was heavily influenced by which serotype-specific antibodies that were used for the evaluation. We found that the arbitrary choices of serotype-specific antibodies could change the fraction of patients deemed to have 'adequate immunity' by a factor of four, exposing an inherent flaw in the guidelines. The flaw relates to dichotomous principles for data interpretation under the AAAAI guidelines. We therefore propose a revised protocol for diagnostic vaccination limited to PPV vaccination, subsequent antibody measurements, and data interpretation using Z-scores. The Z-score compiles multiple individual antibody levels, adjusted for different weighting, into one single continuous variable for each patient. In contrast to interpretation according to the AAAAI guidelines, the Z-scores were robust to variations in the choice of serotype-specific antibodies used for interpretation. Moreover, Z-scores revealed reduced immunity after vaccination in the patients with recurrent pneumonia (a typical symptom of antibody deficiency) compared with control patients. Assessment according to the AAAAI guidelines failed to detect this difference. We conclude that our simplified protocol and interpretation with Z-scores provides more robust clinical results and may enhance the value of diagnostic vaccination.

Keywords: diagnostic vaccination, primary immunodeficiency, antibody deficiency, vaccination, pneumococcal vaccines, z-score, clinical guidelines

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INTRODUCTION

Test of antibody responses to vaccination (diagnostic vaccination) is pivotal in clinical evaluation of patients with suspected antibody deficiency (1–4). A typical symptom of antibody deficiency is recurrent airway infections, although additional infectious disease susceptibilities and comorbidities can be present (5, 6). Assessment of antibody competence is therefore a general recommendation for patients with suspected primary immunodeficiency diseases (7). Although diagnostic vaccination is widely used, the details of the procedure vary (8–11).

Diagnostic vaccination entails measurement of vaccine-specific serum antibodies before and after vaccination. Unconjugated 23-valent pneumococcal capsular-polysaccharide vaccines (PPV) are often used for this purpose. Detailed guidelines for the use of PPV in diagnostic vaccination were proposed in 2012 by the *American Academy of Allergy, Asthma & Immunology* (AAAAI) (7). These guidelines are based on several key concepts (1, 7, 12). First, serum levels of individual serotype-specific antibodies should be quantified before vaccination and four to eight weeks after vaccination. Second, dichotomous principles are recommended for data interpretation: i) antibody levels of 1.3 mg/L or higher are considered 'protective' against a given serotype and ii) adequate antibody immunity in adults requires 'protective' levels for at least 70% of the tested serotype-specific antibodies (7). Crucially, the exact number of serotype-specific antibodies for assessment and their serotype specificities are not defined. However, it is implicit in the guidelines that multiple different serotype-specific antibodies should be tested (7).

Compliance with the AAAAI guidelines thus requires several correctly timed actions: two blood samplings, vaccination with the proper vaccine, measurements of antibody levels using an appropriate assay, and interpretation of immune status according to complicated rules (**Figure 1A**). We hypothesized that strict adherence to the AAAAI guidelines will often fail in actual clinical practice.

The recommended dichotomous approach to the interpretation of diagnostic vaccination is problematic. Such dichotomization introduces a complex, non-monotonic relationship between the probability of qualifying for adequate immunity and the number of tested serotype-specific antibodies (**Figure 1B**) (10). For example: when measuring seven, eight, or nine antibodies, the immunity is deemed 'inadequate' according to the guidelines if at least three antibodies are below the limit of 1.3 mg/L (because fewer than 70% of the antibodies will be 'protective'). However, the probability that at least three antibodies are below 1.3 mg/L obviously increases with the number of antibodies tested. The probability of concluding 'inadequate immunity' in a patient is thus more likely when testing nine antibodies than when testing seven antibodies. A similar principle applies when more antibodies are tested (**Figure 1B**). Another weakness is that different serotype-specific antibodies do not have equal probabilities of reaching a level of at least 1.3 mg/L. In patients with suspected immunodeficiency disease, the mean levels differ for different serotype-specific antibodies (13–15). A limit of 1.3 mg/L regardless of specificity is thus somewhat arbitrary (16) and therefore not necessarily optimal. However, these factors ultimately decide the outcome of diagnostic vaccination, and therefore the clinical evaluation of the individual patient. Moreover, the dichotomous principles hinder comparison of patient cohorts, unless an identical panel of serotype-specific antibodies (and assays) are used.

Diagnostic vaccination using *continuous* variables for interpretation is more attractive from a theoretical standpoint (10). We have proposed using the Z-score, which is more robust

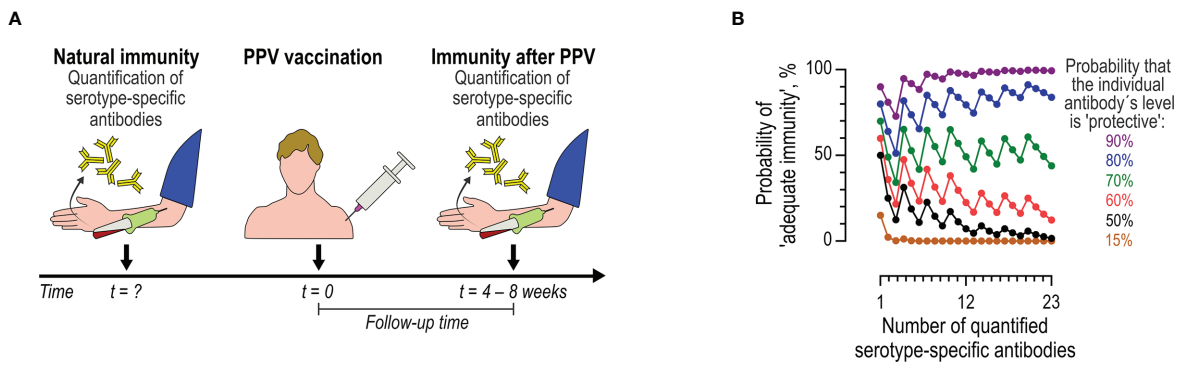

FIGURE 1 | Diagnostic vaccination according to the AAAAI guidelines. **(A)** Flow of events. The preexisting immunity (natural immunity), represented by the levels of multiple (undefined number) serotype-specific antibodies, is determined at an undefined time-point before vaccination (t = ?). Later, PPV is administered (t = 0). The immunity after PPV is assayed four to eight weeks later by quantifying the same serotype-specific antibodies. **(B)** Model showing the theoretical probability of achieving adequate immunity (y-axis, left) according to the AAAAI guidelines (i.e., the probability of at least 70% of serotype-specific antibodies reaching levels of at least 1.3 mg/L) as a function of the number (x-axis) of tested serotype-specific antibodies. The colored curves represent different probabilities of an individual antibody being classified as 'protective' (i.e., a level of at least 1.3 mg/L). The probability of achieving 'adequate immunity' follows the binomial distribution, under the simplifying assumption that the individual serotype-specific antibodies in a given panel have equal likelihoods of being at the 'protective' level (although this will rarely be the case, the simplification nonetheless serves to illustrate the underlying problem).

than the dichotomous assessment to both the number of antibodies and their serotype-specificities (10). The Z-score is based on standard normal deviations of the individual serotype-specific antibodies (**Figure 2**). Because individual standard normal deviations are compiled by a simple mean, the complex relationship between the number of antibodies and the outcome (inherent in the dichotomous approach) is eliminated. A direct comparison of the outcomes of diagnostic vaccination using Z-score and the dichotomous principles is hitherto unreported for patients with suspected primary immunodeficiency diseases.

Our aims were thus i) to evaluate the degree of compliance with AAAAI guidelines for diagnostic vaccination in a tertiary center for primary immunodeficiency diseases and ii) to compare the outcome of diagnostic vaccination based on AAAAI guidelines to that based on Z-scores.

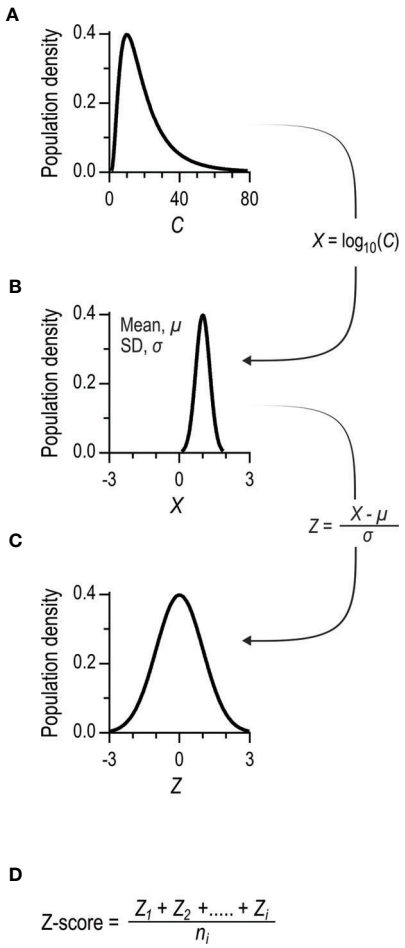

FIGURE 2 | Principles for calculating Z-scores. **(A)** The distribution of serum concentrations of a serotype-specific antibody in a population. The distribution is typically left-skewed. **(B)** Log_{10} transformation of data results in Gaussian distribution. **(C)** The individual concentrations are further transformed to standard normal distributions. This is achieved by subtracting the mean and dividing by the standard deviation of the population dataset. The final parameter is dimensionless, and the population data set has a mean of 0 and a standard deviation of 1. **(D)** The Z-score is calculated for each patient as the mean of the standard normal deviations of the individual antibody levels. The Z-score´s standard deviation tends to decrease with an increasing number of distinct serotype-specific antibody levels, owing to mutual correlations (10). To promote comparability of cohorts tested with different number of measured serotype-specific antibody levels, the Z-score is normalized by the standard deviation of the population dataset.

MATERIAL AND METHODS

Patients

Eligible patients were referred to advanced laboratory evaluation for immunodeficiency at the Department of Clinical Immunology, Aarhus University Hospital, Denmark over a five-year period (from May 2011 to August 2016, n = 687). Only patients referred from the Department of Infectious Diseases, Aarhus University Hospital, Denmark were included in the final cohort (n = 229). In Denmark, diagnostics and treatment of immunodeficiency is part of the general healthcare freely available to all citizens. The Department of Infectious Diseases, Aarhus University Hospital, Denmark is the specialized clinical center covering all adults with primary immunodeficiency diseases living in the Central Denmark Region (1.3 million inhabitants). All included patients were adults suspected of primary immunodeficiency disease by experienced infectious disease clinicians. We categorized patients into infection profiles based on referral data. The patient population consisted of both patients with normal immunoglobulin concentrations and patients with reduced immunoglobulin concentrations. This information was not systematically available for the present study. The patients represented the majority of the patients with idiopathic infections in a recent study (17). **Figure 3** shows a flow chart of the establishment of the final cohort. The predominant reason for exclusion was a lack of increased susceptibility to infections (n = 287).

For the audit, we obtained data on administered pneumococcal vaccines and IgG substitution from medical records. The study was conducted under the approval of the *Ethics Committee in Central Denmark Region* (reference number 1-10-72-127-12), and the Danish Data Protection Agency (reference number 1-16-02-40-12/2007-58-0010) in accordance with Danish legislation.

Antibody Measurements

Evaluation of anti-pneumococcal antibody levels was part of the routine clinical practice at Department of Infectious Diseases, Aarhus University Hospital. Clinicians could choose between qualitative or quantitative antibody assays. For either assay, the concentration of specific IgG antibodies against 12 pneumococcal capsules (serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) were determined in serum samples by an in-house Luminex-based method described by Lal et al. (18). Concentrations (in mg/L) were measured for each of the 12 measured serotype-specific antibodies. For quantitative assays, the concentrations were reported individually for the serotype-

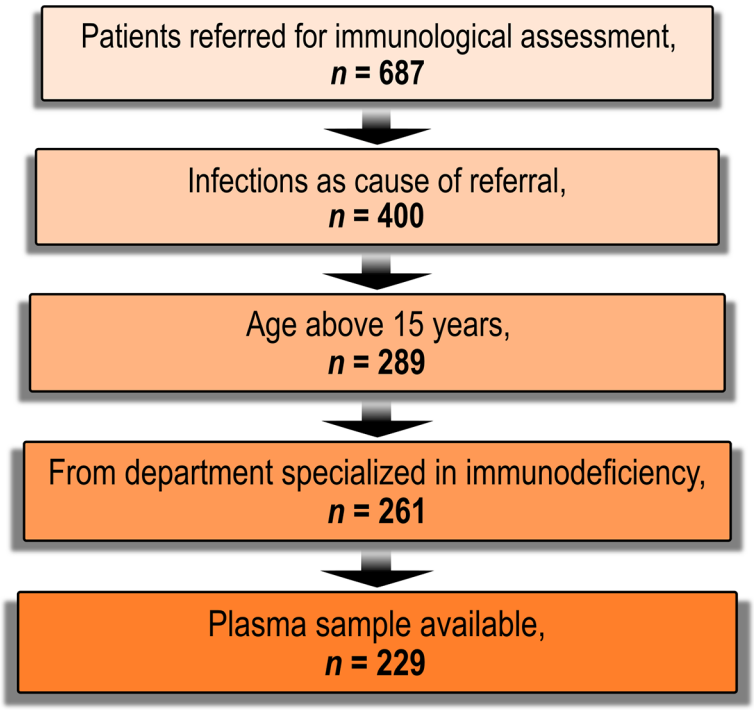

FIGURE 3 | Flow-chart of patient enrolment. Candidates eligible for inclusion were patients referred for advanced laboratory assessment of immunodeficiency at the department of Clinical Immunology, Aarhus University Hospital, Denmark between 12 May, 2011 and 18 August, 2016 (*n* = 687). Patients without an increased susceptibility to infections were excluded (*n* = 287). Patients younger than 16 years were excluded (*n* = 111) as diagnostic vaccination is not local practice in these patients. We also excluded patients who were referred from departments not specialized in immunodeficiency (*n* = 28). Patients without a previously archived plasma sample were also excluded (*n* = 32).

specific antibodies, as required for interpretation with the AAAAI guidelines. Qualitative assays were reported as 'protective immunity' when the geometrical mean of the 12 individual serotype-specific antibodies was at least 1 mg/L (the levels of individual serotype-specific antibodies were not reported); however, such data cannot be interpreted under the AAAAI guidelines. In Denmark, medical data are linked to the individual patient *via* the national Danish Personal Identification Number system. Using the identification numbers of the patients in the final cohort, we retrieved complete data on all measured anti-pneumococcal antibodies with the multiplex assay (preceding April 18, 2018). This also included quantitative data of measurements originally requested as qualitative by the clinicians.

Assessment of Antibody Levels

We excluded measurements from patients who i) had received IgG replacement therapy within the previous six months, or ii) had a history of any pneumococcal vaccination before the study period, or iii) were previously vaccinated with protein-conjugated pneumococcal vaccines during the study period. Conjugate vaccines elicits an antibody response by different mechanisms than do natural infection or PPV vaccination (19), which can affect the response to subsequent vaccination with PPV (1, 7). Each of the 12 serotype-specific antibodies were quantifiable in all available measurements.

Natural immunity was defined as the serotype-specific antibodies that pre-existed before PPV vaccination. For patients with several available antibody measurements, we used the following rules to include a single measurement per patient only: patients who did not receive PPV during the study period had results from their first measurement included; and patients who received PPV during the study period had their results from the last measurement before PPV included. In the subset of patients for investigations of natural immunity, we also included the qualitative data from patients where the clinicians had requested qualitative measurements. The total subset comprised 154 patients.

Immunity after PPV. For patients with several antibody measurements, we selected the first measurement that occurred between four weeks and eight weeks after their PPV vaccination [(in compliance with the AAAAI guidelines (1, 7, 12)]. For patients who only had antibody measurements outside this interval, we included the antibody measurement closest to this interval. In the subset of patients for investigations of immunity after PPV vaccination, we also included the qualitative data from patients where the clinicians had requested qualitative measurements. The total subset comprised 98 patients.

Assessment by dichotomous principles. 'Protective level' for individual serotype-specific antibodies was defined as at least 1.3 mg/L in agreement with AAAAI guidelines (1, 7, 12). 'Adequate immunity' was defined as at least 70% of the interpreted serotype-specific antibodies having 'protective levels'.

Z-scores were calculated as previously described (10). The principles are summarized in **Figure 2**. Briefly, the levels of individual serotype-specific antibodies were transformed to standard normal distributions. For each patient, the Z-score was the average of the standard normal deviation of each of the twelve individual antibodies. A Z-score (also called a standard score thus represents how many standard deviations a raw score is from the population mean.

Statistics

We estimated 95%-confidence intervals (reported in square brackets) for effect sizes using Estimation Statistics (www.estimationstats.com) (20) and for means using t-distributions (continuous variables) or exact binomial statistics (dichotomous variables). STATA 11 (StataCorp LP, TX, USA) was used for data analysis other than estimations of effect sizes. Given the exploratory nature of our study, we refrained from making corrections for multiple comparisons (to limit risk of type II errors). To limit the risk of type I errors, we minimized the number of comparisons to those deemed strictly relevant. When more than two groups were available for comparisons, we therefore defined one shared control group. Graphs were made in GraphPad PRISM v. 6.07 (GraphPad Software, CA, USA). The level of significance was defined as 0.05.

RESULTS

Study Population

The total cohort comprised 229 patients (**Figure 3**). Recurrent respiratory tract infections (a cardinal sign of antibody deficiency) was the predominant type of infection in 142 patients. These patients were subdivided into those with increased tendency to lower-respiratory tract infections ('LRTI', n = 114) and those with increased tendency to upper-respiratory infections without increased tendency to lower airway infections ('URTI', n = 28). The remaining 87 patients, labeled 'control', suffered from other types of infections (**Table 1**) that did not elicit suspicion of antibody deficiency. In the final cohort, 73% were female. The median age was 50 years (range 16 to 83 yrs.). Patients in the LRTI group were on average 11 [6; 15] years older than controls. The age of the patients in the URTI group was comparable to that of the control group.

Compliance With the Guidelines for Diagnostic Vaccination

For our evaluation of compliance with the AAAAI guidelines in clinical practice, we categorized the patients as those with i) failed initiation, ii) failed procedure, and iii) completed procedure (**Table 2**).

Failed initiation was concluded for patients where anti-pneumococcal antibodies were never measured. This applied to 47 cases (21%). The finding was more common in the control group, 46%, compared with the LRTI group, 5.3% (difference -41% [-52%; -29%], i.e., 9-fold difference), and 3.6% in the URTI group (difference -42% [-52%; -25%], i.e., 13-fold difference).

Failed procedure was concluded for patients where the procedure had been commenced, (i.e., the antibodies had been quantified) but the available data were insufficient for interpretation under AAAAI guidelines. This applied to most patients (62%). Of the commenced procedures (n = 182), no group difference was found for failure frequency: 79% in the control group, 80% in the LRTI group (difference 0.91% [-13%; 16%]), and 74% in the URTI group (difference -4.7% [-27%; 14%]). The reason for failed procedure differed. The most common cause was a request of qualitative antibody assay instead of the required quantitative assay. This accounted for 43% of all failed procedures. We compared the outcome of the available qualitative assay ('protective immunity' defined as a geometrical mean of individual serotype-specific antibodies of at least 1 mg/L) with outcomes based on the AAAAI guidelines. The former concluded four times as many of the evaluations as 'protective immunity' than the latter (**Figure S1**). The second most frequent cause of failed procedure was a lack of PPV vaccination despite proven inadequate natural immunity. This explained 25% of the failed procedures. The remaining causes of failed procedures are given in **Table 2**.

Completed diagnostic vaccination was concluded for patients with i) documented adequate natural immunity or ii) documented inadequate natural immunity followed by PPV vaccination and quantification of serotype-specific antibodies four to eight weeks later. This applied to 39 patients (17%) only. Of the commenced procedures (n = 182), 21% were completed overall. No group difference was found: 21% in the control group, 20% in the LRTI group (difference -0.91% [-16%; 12%]), and 26% in the URTI group (difference 4.7% [-14%; 26%]).

TABLE 1 | Patients categorized by their dominating type of infections.

	Case patients				Control patients										All patients	
	LRTI		URTI		Abscesses		Viral		Fungal		Invasive bacterial		Other			
Number	114		28		39		27		6		5		10		229	
Female, *n* (%)	87	(76)	23	(82)	27	(69)	20	(74)	2	(33)	4	(80)	5	(50)	168	(73)
Median age, yrs. (range)	57	(18–83)	45	(21–76)	39	(22–76)	43	(16–68)	55	(45–63)	40	(21–63)	56	(20–67)	50	(16–83)

LRTI: Patients with reported increased tendency to lower-respiratory tract infections.

URTI: Patients with reported increased tendency to upper-respiratory tract infections without reported increased tendency to lower-airway infections.

TABLE 2 | Compliance with the AAAAI guidelines for diagnostic vaccination.

	Case patients				Control patients										All patients,	
	LRTI, *n* = 114		URTI, *n* = 28		Abscesses, *n* = 39		Viral, *n* = 27		Fungal, *n* = 6		Invasive bacterial, *n* = 5		Other, *n* = 10		*n* = 229	
Failed initiation (serotype-specific antibodies not measured), ***n*** **(%)**	**6**	**(5.3)**	**1**	**(3.6)**	**13**	**(33)**	**16**	**(59)**	**2**	**(33)**	**4**	**(80)**	**5**	**(50)**	**47**	**(21)**
Failed procedure, *n* (%)	**86**	**(75)**	**20**	**(71)**	**20**	**(51)**	**9**	**(33)**	**3**	**(50)**	**1**	**(20)**	**4**	**(40)**	**143**	**(62)**
Serotype-specific antibody measurements:																
Never quantitative	22	(19)	1	(3.6)	3	(7.7)	7	(26)	1	(17)	0		1	(10)	35	(15)
Lacking before vaccination	16	(14)	6	(21)	3	(7.7)	0		0		0		1	(10)	26	(11)
Only qualitatively before vaccination	5	(4.4)	2	(7.1)	1	(2.6)	0		0		1	(20)	2	(20)	11	(4.8)
Natural immunity inadequate but:																
PPV not administered	14	(12)	9	(32)	9	(23)	2	(7.4)	2	(33)	0		0		36	(16)
PPV administered but follow-up antibody measurements:																
Lacking	4	(3.5)	1	(3.6)	1	(2.6)	0		0		0		0		6	(2.6)
Qualitative	14	(12)	0		1	(2.6)	0		0		0		0		15	(6.6)
Quantitative but before week 4	1	(0.88)	0		0		0		0		0		0		1	(0.44)
Quantitative but after week 8	7	(6.1)	0		1	(2.6)	0		0		0		0		8	(3.5)
Date of PPV vaccination uncertain	3	(2.6)	0		0		0		0		0		0		3	(1.3)
Previous administered conjugate pneumococcal vaccine	0		1	(3.6)	1	(2.6)	0		0		0		0		2	(0.87)
Completed procedure, *n* (%)	**22**	**(19)**	**7**	**(25)**	**6**	**(15)**	**2**	**(7.4)**	**1**	**(17)**	**0**		**1**	**(10)**	**39**	**(17)**
Adequate natural immunity, PPV not administered	1	(0.9)	2	(7.1)	3	(7.7)	1	(3.7)	1	(17)	0		0		8	(3.5)
Adequate natural immunity, PPV administered (superfluous)	0		1	(3.6)	0		1	(3.7)	0		0		0		2	(0.87)
Inadequate natural immunity, PPV administered and follow-up with quantitative antibody measurements	21	(18)	4	(14)	3	(7.7)	0		0		0		1	(10)	29	(13)

Each participant was assigned to the first correct category in the left column (top-to-bottom). Adequate natural immunity was defined as ≥ 70% of measured levels of serotype-specific antibodies ≥ 1.3 mg/L.

Categories of compliance are indicated in bold.

In conclusion, compliance with the AAAAI guidelines seems difficult to accomplish in routine settings. Different meticulousness among physicians could be an important factor. In our setting, two clinicians were responsible for 93% of the referred patients. Clinician A referred 115 of the patients and completed the procedure for 23% of these. Clinician B referred 97 of the patients and completed the procedure for 11% of these (i.e., two-fold lower completion frequency). Thus, even among experienced clinicians, the chance of completing diagnostic vaccination according to the AAAAI guidelines varies markedly.

Levels of Serotype-Specific Antibodies in the Cohort

We claim that the outcome of diagnostic vaccination conducted under AAAAI principles is influenced by the choice of antibody specificities for evaluation. The various antibodies cannot be expected to have the same probability of achieving a concentration of at least 1.3 mg/L. We examined this in details for the cohort.

The natural immunity (pre-existing antibody levels) could be assessed for 154 of the patients. This included data from assays originally requested as qualitative. We found that the antibody levels differed markedly between different serotype-specificities (**Figure 4A**). The mean level differed approximately ten-fold between anti-serotype 3 antibody (0.25 mg/L) and anti-serotype 23F antibody (2.3 mg/L). Anti-serotype 4 antibody showed the least variation between patients (480-fold) and anti-serotype 5 antibody showed the greatest variation (15,000-fold). The levels of the 12 antibody specificities correlated positively in the patients, e.g., the correlations between anti-serotype 4 antibody levels and the levels of each of the other 11 antibodies displayed Spearman's ρ of minimum 0.30 ($p \leq 0.0001$). As expected, the proportion of patients with levels of at least 1.3 mg/L differed markedly between the different serotype-specific antibodies (**Figure 4B**). The proportion differed as much as five-fold between anti-serotype 3 antibody (12%) and anti-serotype 23F antibody (66%).

For assessment of the immunity after PPV vaccination, antibody quantifications were available for 98 patients. The follow-up time was between four and eight weeks for 72% of the patients (median 5 weeks), shorter for 6.5% (median 3 weeks), and longer for 22% (median 19 weeks). We found that the mean antibody levels differed markedly for the different serotype specificities after vaccination (**Figure 4C**). The mean level differed approximately 10-fold between anti-serotype 3 antibody (0.37 mg/L) and anti-serotype 14 antibody (3.6 mg/L). Anti-serotype 4 antibody showed least variation between patients (380-fold) and anti-serotype 23F antibody showed most variation (12,000-fold). The antibody levels of the 12 specificities correlated positively in the individual patients, e.g., the correlations between anti-serotype 4 antibody

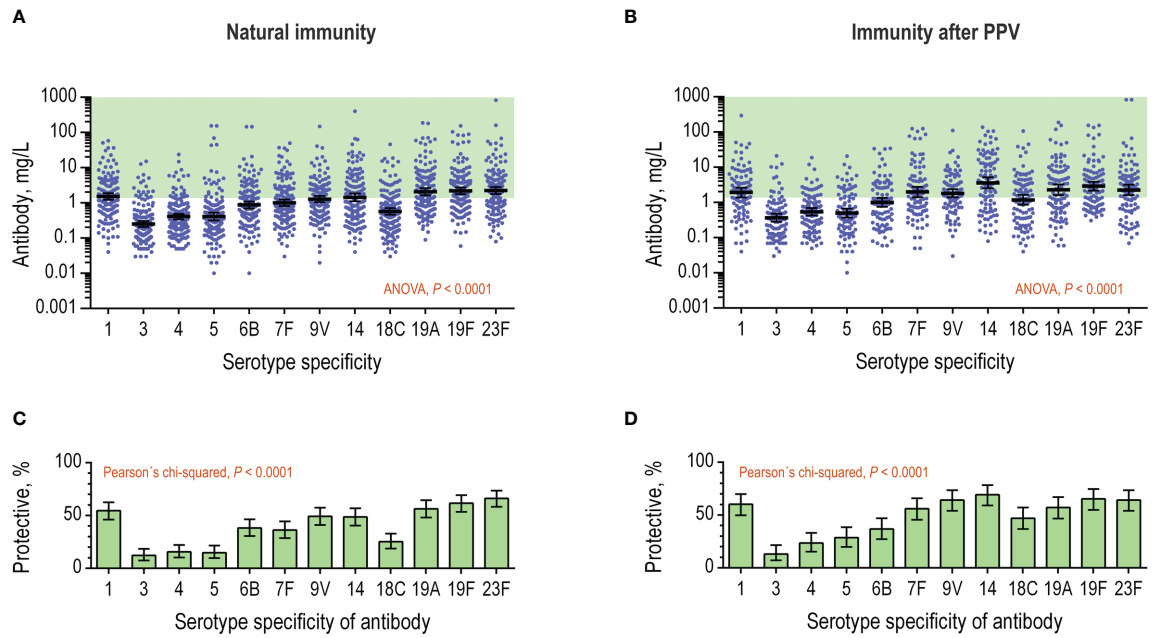

FIGURE 4 | Serotype-specific antibody levels in the patient serum samples. The concentrations (mg/L) of 12 different antibodies were determined in serum samples by a multiplex, bead-based assay. **(A)** The natural immunity of the patients (n = 154) displayed for each of the antibodies as continuous variables. The green area indicates concentrations of at least 1.3 mg/L, which is considered as 'protective' under AAAAI guidelines. Error bars are geometrical means with 95% confidence intervals. The antibodies were tested for different levels using repeated-measures ANOVA. **(B)** Data from previous panel showing the proportion of patients with antibody levels of at least 1.3 mg/L (i.e., 'protective level') for each specific antibody. **(C)** As in the panel A, but for levels measured in serum samples collected after PPV vaccination (n = 98). **(D)** Data from previous panel, showing the proportion of patients with antibody levels of at least 1.3 mg/L for each specific antibody.

and each of the other 11 antibody specificities displayed Spearman's ρ of minimum 0.30 ($p \leq 0.0029$). Again, the proportion of patients with antibody levels of at least 1.3 mg/L differed markedly between the different serotype-specificities (**Figure 4D**). The proportion differed as much as 5-fold between anti-serotype 3 antibody (13%) and anti-serotype 14 antibody (69%).

The collective results confirm that concentrations differ between different serotype-specific antibodies in patients with suspected primary immunodeficiency diseases. In addition, serotype-specific antibodies show different probabilities for fulfilling the criterion for a protective level as defined in the AAAAI guidelines.

Dichotomous Assessment of Diagnostic Vaccination Is Not Robust

We explored how the different probabilities of reaching 1.3 mg/L among different serotype-specific antibodies affected the outcome of diagnostic vaccination based on the AAAAI guidelines. Specifically, we separated the 12 measured serotype-specific antibodies into different arbitrary antibody panels (I, II, III, and IV) and compared the outcomes. The four panels each contained measurements of six different antibody specificities to eliminate the effect of different antibody numbers (**Figure 1B**). Partial combinations of the measured antibody specificities were used for this particular sensitivity analysis only. In all other analyses, all antibody specificities measured were applied. For this analysis, we used the available antibody measurements obtained after PPV vaccination (n = 98).

We first examined a worst-case scenario by comparing the outcomes of two antibody panels (I and II). Panel I contained the six antibody specificities with the highest proportion of levels at or above 1.3 mg/L (i.e., anti-serotype 14, -19F, -9V, -23F, -1, and -19A). Panel II contained the six antibodies with the lowest proportion of levels at or above 1.3 mg/L (anti-serotype 7F, -18C, -6B, -5, -4, and -3). Analysis using panel I resulted in 'adequate immunity' for 42% of the patients and analysis using panel II resulted in 'adequate immunity' for only 10% of the patients (paired mean difference 32% [-43%; -24%]) (**Figure 5A**). The arbitrary choice of serotype-specific antibodies in the two panels thus resulted in a four-fold difference in the proportion of patients with adequate immunity under AAAAI guidelines. Next, we made a recalculation based on the Z-score approach. The Z-score is theoretically more robust across interpretation of different serotype-specific antibodies (10). To test this in practice, we compared the mean Z-scores for panel I and II. As anticipated, no systematic difference in Z-score was found when using either panel (paired mean difference 0.0 [-0.12; 0.14]) (**Figure 5B**). Consistency of the outcome is also important. According to the AAAAI guidelines, 41 patients had adequate immunity in panel I but only nine of these patients (22% [9.8%; 34%]) also displayed adequate immunity in panel II (**Figure 5C**). Ten patients had adequate immunity in panel II and nine of these

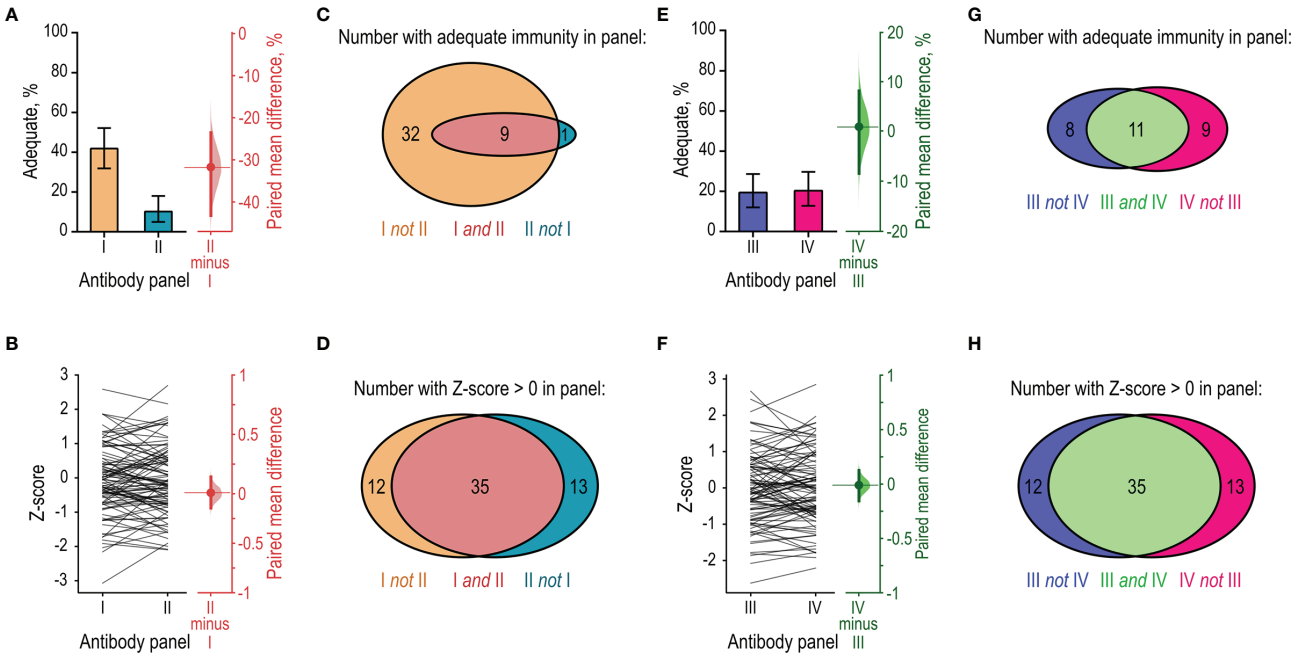

FIGURE 5 | Sensitivity-analysis of interpretation using the AAAAI guidelines and Z-scores. The same cohort of patients (*n* = 98) were assessed for adequate immunity after PPV vaccination using different panels of serotype-specific antibodies for the interpretation. Each panel contained six out of the 12 quantified serotype-specific antibodies. **(A)** Left: The proportions of patients considered to have achieved 'adequate immunity' in each of two antibody panels (I and II) according to the AAAAI guidelines. Right: The paired mean difference in proportions (dot) with 95% confidence interval (vertical error lines) and bootstrap sampling distribution (normalized histogram). Panel I contained the six serotype-specific antibodies that most frequently had levels of 1.3 mg/L or higher in the patients. Panel II contained the six serotype-specific antibodies that least frequently had levels of 1.3 mg/L or higher in the patients. **(B)** Left: Paired Z-scores for the individual patients calculated using data from panel I and panel II. Right: The paired mean difference in Z-score from the two panels. **(C)** Venn-diagram showing the number of patients with adequate immunity in panels I and II under AAAAI guidelines. Panels I and II identified an unequal numbers of the patients, and the results showed little overlap. **(D)** As in **(C)**, but for Z-scores. Panels I and II now identified comparable numbers of the patients, and the results largely overlapped. **(E)** As in **(A)**, but for two other panels (III and IV). These panels were composed to provide an equal proportion of patients with 'adequate immunity' under AAAAI guidelines. **(F)** Comparison of the Z-scores calculated for the patients using the data from panels III and IV. **(G)** Venn-diagram showing the number of patients with adequate immunity in panels III and IV according to the AAAAI guidelines. Panels III and IV identified comparable numbers of the patients, but the results showed little overlap. **(H)** As in **(G)**, but for Z-scores. Panels III and IV identified comparable numbers of the patients, and the results largely overlapped.

patients (90% [50%; 100%]) also showed adequate immunity in panel I (**Figure 5C**). For the Z-score, the limit for adequate immunity is not yet defined. To facilitate a comparison of Z-score consistency between antibody panels, we assigned an arbitrary cutoff of 0. Forty-seven patients had Z-score above 0 in panel I and 35 of these patients (76% [59%; 84%]) also had Z-score above 0 in panel II (**Figure 5D**). The reverse comparison gave similar results (**Figure 5D**). Thus, in the worst-case scenario, interpretation based on Z-score was markedly less sensitive to the choice of serotype-specific antibodies than the AAAAI guidelines.

We then examined a best-case scenario by constructing two other antibody panels (III and IV) of similar propensity for achieving 'adequate immunity' under the AAAAI guidelines. To design these panels, we ranked the twelve serotype-specific antibodies according to their frequency of being at least 1.3 mg/L. Panel III contained the six antibodies with rank numbers 1, 4, 5, 8, 9, and 12 (i.e., anti-serotype 14, -23F, -1, -18C, -6B, and -3). Panel IV contained the six antibodies with rank numbers 2, 3, 6, 7, 10, and 11 (i.e., anti-serotype 19F, -9V, -19A, -7F, -5, and -4). As intended, a similar proportion of the patients had adequate immunity according to the AAAAI guidelines in the two panels (**Figure 5E**). Z-scores were also similar (**Figure 5F**). According to the AAAAI guidelines, 19 patients had adequate immunity in panel III but only 11 of these patients (58% [26%; 74%]) also had adequate immunity in panel IV (**Figure 5G**). Twenty patients had adequate immunity in panel IV but only 11 of these patients (55% [25%; 70%]) also had adequate immunity in panel III (**Figure 5G**). However, compared to interpretation according to the AAAAI guidelines, significantly better consistency was achieved when data from panels III and IV were interpreted with Z-scores (**Figure 5H**). With the Z-scores, the inconsistency between panels III and IV corresponded to that observed between panels I and II (cf. **Figures 5D, H**).

The collective results support the conclusion that the Z-score provides more robust results than the AAAAI guidelines.

Natural Immunity to Pneumococci in Patient Subgroups

Next, we compared the two approaches for interpretation in different patient subgroups. The subgroups were defined

according to infection profile, gender, and age groups. All measured serotype-specific antibodies were included in a single panel in this part of the study. First, we examined the natural immunity in all patients (n = 154). Overall, 12% [7.6%; 19%] had adequate immunity according to the AAAAI guidelines.

When divided by infection profile, the control group (n = 42) displayed higher mean Z-score than patients in the LRTI group (n = 91) (difference -0.38 [-0.81, -0.0025]) (**Figure 6A**). No significant difference was observed between the control group and the URTI group (n = 21). The dichotomous approach also identified more frequent adequate immunity in the control group, 19%, compared with patients in the LRTI group, 7.7% (difference -11% [-27%; -0.55%]) (**Figure 6B**). Similarly, this approach did not identify a significant difference between patients in the control group and the URTI group.

Neither of the two approaches identified any difference according to gender (**Figures 6C, D**).

The relationship between age and Z-score showed an inverted, flattened U-shaped relationship, with Z-scores peaking in the age-group 30 to 50 years (**Figure S2**). Compared with patients in the age-group 30 to 50 years (n = 53), younger patients (n = 24) and older patients (n = 77) had lower mean Z-score (difference -0.55 [-0.98; -0.097] and -0.46 [-0.86, -0.13]) (**Figure 6E**). Adequate immunity according to the AAAAI guidelines also peaked in the age-group 30 to 50 years (**Figure S3**). However, the dichotomous approach did not detect statistical differences relating to age groups (**Figure 6F**).

In summary, the estimated natural immunity was weaker in patients with recurrent LRTI compared with the control group. The Z-score, but not the AAAAI guidelines, identified stronger natural immunity in patients aged 30 to 50 years compared with both younger and older patients. Gender was not associated with any difference in natural immunity.

Immunity After PPV Vaccination in Patient Subgroups

We compared the outcomes of the two approaches for interpreting immunity after PPV vaccination in patient subgroups (n = 98). Overall, 23% [15%; 33%] had adequate immunity based on the AAAAI guideline principles.

When divided according to infection profile, the control group (n = 16) had a higher mean Z-score than the LRTI group (n = 70) (difference -0.72 [-1.1; -0.25]) (**Figure 7A**). No significant difference was observed between the control group and the URTI group (n = 12). However, interpretation with the dichotomous approach did not detect significant differences relating to infection subgroups (**Figure 7B**).

Neither approach for interpretation identified any significant differences relating to gender or age groups (**Figures 7C–F**). Increasing age did, however, show a tendency towards decreasing immunity (**Figures S4, S5**).

In contrast to interpretation under AAAAI guidelines, the Z-score identified weaker immunity after PPV in the LRTI patient group compared with the control group. None of the approaches for interpretation detected significant differences in immunity related to gender or age.

DISCUSSION

This study demonstrates two significant caveats in diagnostic vaccination based on the AAAAI guidelines. First, correct procedure may be difficult to achieve in routine clinical settings. Second, the dichotomous principle applied for result interpretation introduces complex effects of two arbitrary choices, namely the number and the serotype-specificity of the quantified antibody levels. In contrast, evaluation based on continuous variables, such as Z-scores, may simplify the procedure and add robustness. In our study, Z-scores after PPV vaccination discriminated patients with recurrent lower respiratory tract infections from patients with infections that did not evoke suspicion of antibody deficiency. The AAAAI guidelines failed in detecting this difference.

The retrospective design of this study is a strength in providing unbiased data for our audit of diagnostic vaccination in routine clinical practice. A monitored clinical trial likely offers less valid data for an audit. A further asset of the study is that a single laboratory performed all antibody measurements using the same assay, which adds comparability to the data set. The use of a single statistical approach for determining effect sizes for the dichotomous variable and the continuous variable also aids comparability.

This study had limitations. The interpretations made by the individual clinicians were not studied, mainly because clear statements on these matters were rarely provided in the medical records. We therefore cannot rule out that erroneous data interpretation by the clinicians may have reduced the actual frequency of correct procedures to less than the 17% we report. We did not include intentional deviations from the guidelines in our audit. For instance, it is local practice to refrain from diagnostic vaccination of the rare patients with very low plasma IgG concentration (below 1–2 g/L) and symptoms prompting immediate IgG replacement therapy. Also, baseline antibody level measurements are required under AAAAI guideline but were omitted for several patients undergoing diagnostic vaccination. In most cases, this was likely a deliberate choice, insofar as the local clinicians find the absolute antibody concentrations after vaccination of direct interest. The study design and available data do not allow confident conclusions on the underlying cause of the high rate of failure. We do, however, suspect that the complexity of the AAAAI guidelines is responsible. Data on other laboratory parameters, such as the levels of total IgG and IgG subclasses, were not included. We find that such data would not contribute to this head-to-head comparison of methods for assaying diagnostic vaccination. Others have reported a lack of association between these parameters and the outcome of diagnostic vaccination (21). We find that the number of patients included in the study, although limited, is sufficient to assess the applicability of a diagnostic test intended for use in the evaluation of individual patients.

We found it relevant to examine the actual conductance of diagnostic vaccination. Meticulous guidelines are of little use if they are virtually impossible to comply with in clinical practice. Strict interpretation in agreement with recommendations of the

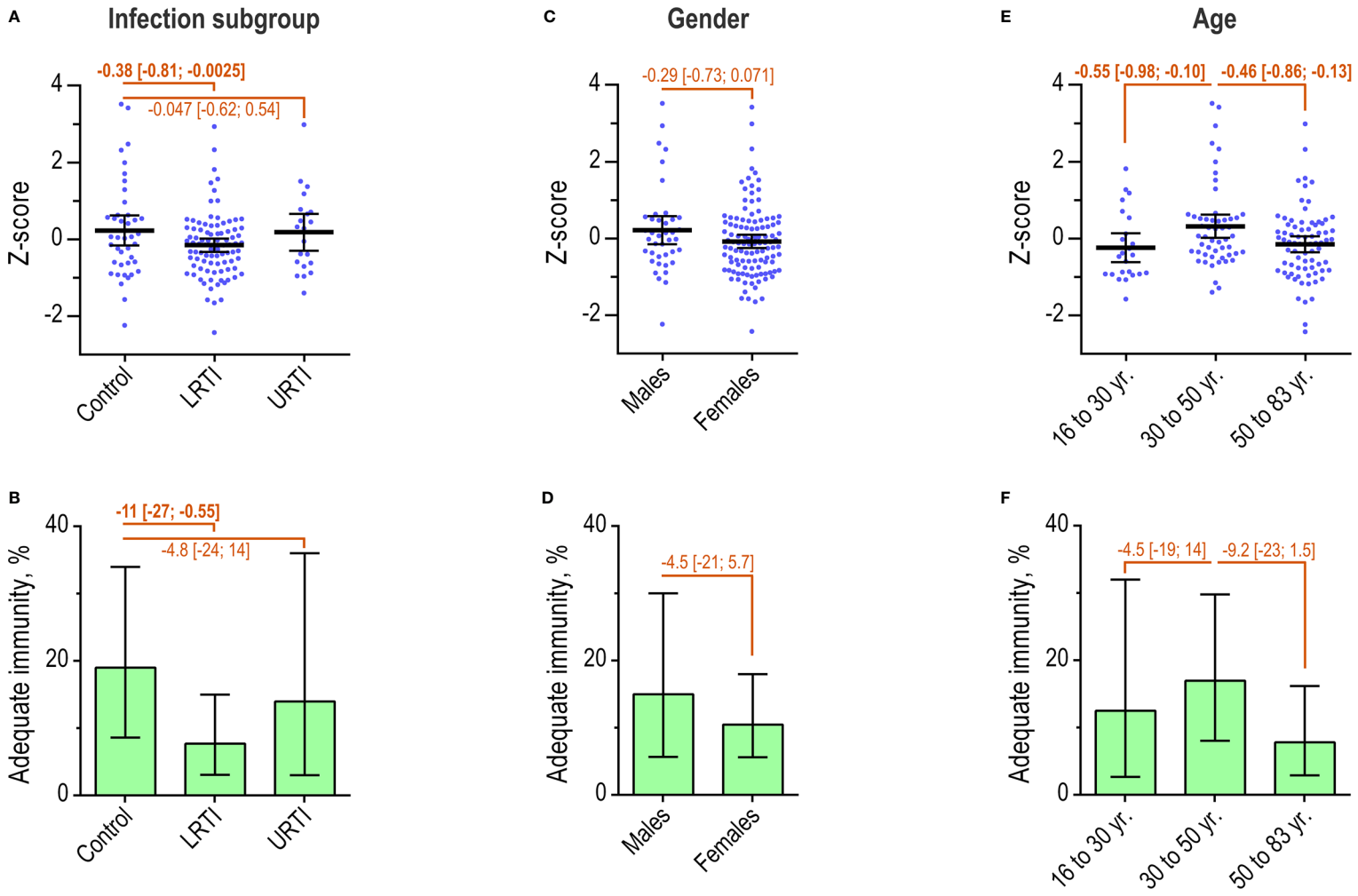

FIGURE 6 | Natural immunity in patient subgroups. **(A)** Individual Z-scores and mean with 95% confidence interval for patients (n = 154), by infection profiles. Differences between groups were determined as indicated. **(B)** The percentage of patients with 'adequate' immunity according to the AAAAI guidelines, by infection profiles. Error bars are 95% confidence intervals. **(C)** As in A, but for patients by gender. **(D)** As in B, but for patients by gender. **(E)** Individual Z-scores for patients by age intervals. **(F)** As in B, but for patients by age group.

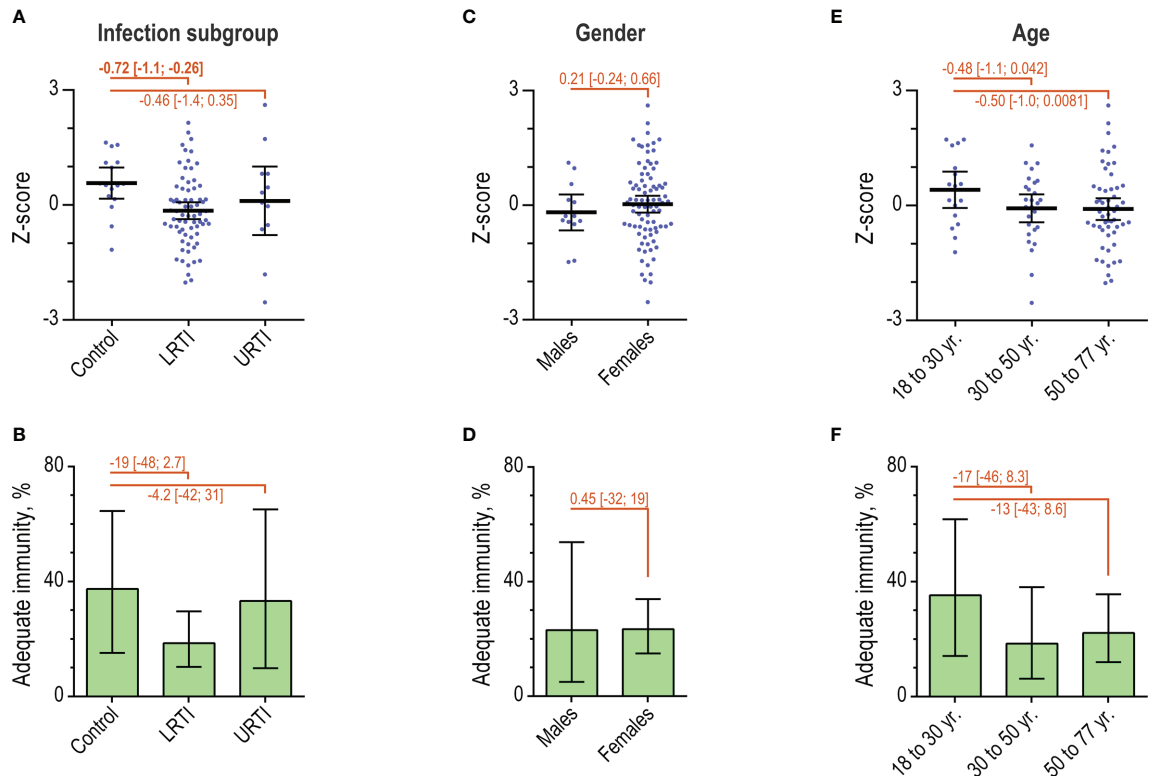

FIGURE 7 | Immunity after PPV in patient subgroups. **(A)** Individual Z-scores and mean with 95% confidence interval for patients (n = 98) by infection profiles. Differences between groups were determined as indicated. **(B)** The percentage of patients with 'adequate' immunity according to the AAAAI guidelines, by infection profiles. Error bars are 95% confidence intervals. **(C)** As in A, but for patients by gender. **(D)** As in B, but for patients by gender. **(E)** Individual Z-scores for patients by age intervals. **(F)** As in B, but for patients by age group.

AAAAI guidelines was possible in only one out of six patients. Our design does not allow us to infer that this is a general trend. But we see no reason to suspect that our observed compliance is especially poor compared with that of other centers. Indeed, there are indications that the procedure also fails frequently elsewhere. For example, Barton and coworkers reported that for 14 out of their 18 patients with IgG2 subclass deficiency, historical data were insufficient to interpret diagnostic vaccination under AAAAI guidelines (22). We suspect that the complexity of the AAAAI guidelines is responsible for the high failure frequency.

Based on the AAAAI criteria, adequate immunity was present in 23% of our patients after vaccination. This is low compared with the frequency reported for some cohorts [typically at least 50% (15, 23–25)] although some studies report similar results (26, 27). Several factors may explain the different findings. The AAAAI guidelines are inherently unreliable for comparing cohorts tested with different panels of serotype-specific antibodies (**Figures 1B**, **5**) (10). Also, use of different assays for antibody quantification is problematic because of poor inter-assay comparability (28–31). Cohorts are likely to differ in their ability to respond to vaccination. Our cohort, comprised of patients referred to advanced laboratory tests for primary immunodeficiency diseases, may be less capable of producing specific antibodies than the majority of reported cohorts.

Both the natural immunity and the immunity after PPV are reported as lower in adults with recurrent lower respiratory tract infections compared with healthy controls (32). In support of such reports, we found that assessment using the AAAAI principles as well as Z-scores identified lower natural immunity in the LRTI group compared with the patient controls suffering from infections that do not indicate antibody deficiency. However, only interpretation based on Z-scores revealed lower immunity after PPV in the LRTI group compared with the patient controls, whereas interpretation with the AAAAI guidelines failed to demonstrate this difference. Estimates based on Z-scores thus seem more sensitive for detection of differences in antibody immunity between patient groups.

Recurrent URTI may also be a sign of antibody deficiency. However, neither approach detected lower immunity in such patients. Our study included few patients with URTI (n = 12 for assessment of immunity after PPV vaccination) and therefore has low statistical power for assessing this issue. We therefore cannot rule out lower immunity in patients with recurrent URTI.

Z-scores detected higher natural immunity in patients aged 30 to 50 years compared with younger as well as older patients. This was not detected by the dichotomous assessment. We speculate that the lower immunity is explained by fewer previous natural immunizing events in the younger patients and by waning immunity in the older patients, in agreement with the general view in the field (33).

We anticipate that adopting continuous variables for assaying diagnostic vaccination can improve interpretation of diagnostic vaccination. The change will also allow better comparison of different cohorts, especially when different numbers of antibodies and different serotype-specific antibodies are tested. Interpretation based on dichotomous principles is inherently sensitive to differences in these factors (see *Introduction* and **Figure 1B**), whereas interpretation based on continuous interpretation is more robust (10). Another shortcoming of the dichotomous principles is that the individual antibody measurement is reduced to an "all-or-nothing" outcome, which reduces information and over-emphasizes trivial differences in concentrations near the cut-off. For example, the difference between an antibody concentration of 1.3 mg/L and 1.2 mg/L is unlikely to be of clinical relevance, yet one is deemed protective whereas the other is not. The Z-score is thus more robust to interpretation under different antibody panels than dichotomous outcomes based on the AAAAI guidelines (**Figure 5**). Moreover, we expect that Z-scores will provide more consistent results across laboratories than the AAAAI guidelines, even when the same antibody specificities are tested. Different laboratories may estimate the concentration of a given antibody specificity very differently (28–31), which is a strong disadvantage for interpretation with the AAAAI guidelines. Such inter-laboratory differences are less critical for Z-scores, as long as the concentration estimates show good correlations. This should be examined in future studies.

To simplify the practical procedure, we propose to omit antibody quantification before vaccination and limit the future protocol to the following:

1. PPV vaccination of the patient.
2. Follow-up blood sample after four to eight weeks only.
3. Quantification of the levels of individual serotype-specific antibodies.
4. Calculation of the patient Z-score by the laboratory.
5. Data evaluation.

The protocol can be used with other polyvalent vaccines and for multiple monovalent vaccines that are administered simultaneously. The proposed four to eight weeks interval for blood sampling simply complies with the AAAAI recommendations for diagnostic vaccination with PPV. This recommendation does not appear supported by data (7), suggesting that the timing may potentially be optimized. We propose to quantify at least six different serotype-specific antibodies, based on previous findings on the relationship between result variations and the number of tested antibodies with the Z-score (10). The calculation of Z-scores requires data on the antibody levels in a suitable reference population such as healthy persons. Z-scores of patients can be interpreted relative to the fraction of the reference persons with equal or lower Z-scores. The estimations may apply the probability density function for the standard normal distribution or a non-parametrical approach.

Although promising, the Z-score approach is not yet ready for clinical application, but requires further study. The suggested protocol should thus be tested and optimized further based on the findings in different patient cohorts and by different laboratories. We plan a retrospective study of the proposed protocol, which will include patients referred to our institution from the end of the inclusion for the present study (August 2016) to the present day. However, prospective studies of the Z-score approach are highly desirable before possible dissemination into clinical practice.

Entirely different approaches for assessing the antibody competence of patients may also be of clinical interest. We recently reported that the level of naturally occurring antibodies against terminal galactose-α-1,3-galactose (anti-αGal) predicts the outcome of diagnostic vaccination in HIV infected adults (34). Anti-αGal antibodies are of particular interest in patients with suspected antibody deficiency. The level of anti-αGal antibodies is low in such patients (17, 35, 36). In humans, the anti-αGal antibodies seem important by targeting various common pathogens (17, 37), leading to activation of immunological effector mechanisms (17, 38), and ultimately protection (17). Future studies may therefore examine the association between the anti-αGal antibodies and vaccine response in patients with suspected primary antibody deficiency.

In conclusion, patients may benefit from revised protocols for the conductance and interpretation of diagnostic vaccination. We provide evidence suggesting that the AAAAI guidelines for diagnostic vaccination are difficult to apply in clinical practice. Even when executed in accordance with guidelines, the categorical interpretation of results remains problematic. We therefore propose that a more pertinent evaluation is achievable with Z-scores, which may also simplify the procedure of diagnostic vaccination.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee in Central Denmark Region Regionssekretariatet Juridisk Kontor Skottenborg 26 8800 Viborg Denmark. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AH and JB conceived the study. AS, CL, and CJ provided data. JB performed data analyses. CJ and CL provided intellectual inputs. AH, MP, and JB wrote the manuscript. All authors edited and approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Establishing the Molecular Diagnoses in a Cohort of 291 Patients With Predominantly Antibody Deficiency by Targeted Next-Generation Sequencing: Experience From a Monocentric Study

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Predominantly antibody deficiencies (PAD) are a heterogeneous group of disorders characterized by dysfunctional antibody production, low immunoglobulin levels in serum and impaired vaccine responses. The clinical picture is variable, ranging from mild symptoms to severe complications, which may include autoimmunity, gastrointestinal disease, allergy, and malignancies. If left untreated, PAD patients are at risk of enduring disease progression, irreversible organ damage, and reduced life expectancy. A timely diagnosis has been shown to significantly improve disease prognosis. Here, we report on our experience using targeted gene panel sequencing by employing Agilent's HaloPlex or SureSelect and Illumina's MiSeq technologies in a cohort of 291 individuals who presented with low or absent immunoglobulin levels in combination with or without other clinical features. In total, we have detected over 57 novel or previously reported relevant mutations in *ADA, ADA2, BTK, CTLA4, LRBA, NFKB1, NFKB2, PIK3CD, STAT3*, and *TNFRSF13B*. Overall, a genetic diagnosis could be made in 24.7% of the investigated patients. The percentage of coverage for the targeted regions ranged from 90% to 98% in this study. Moreover, functional assays were performed on a defined group of the patients carrying candidate variants in *CTLA4*, *LRBA*, *NFKB1* and *BTK*, which confirmed their deleterious effect on protein expression and/or function. This study reiterates that the immunological heterogeneity of predominantly antibody deficiencies may have a diverse genetic origin, although certain

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clinical features may hint towards a specific group of defects. Employing targeted sequencing panels proves to be a very time- and cost-efficient, yet reliable, method for the establishment of a genetic diagnosis in individuals with PAD. However, in case of negative panel results, or if functional testing reveals inconspicuous observations in patients with a clear indication for genetic testing, further work-up including whole exome or whole genome sequencing should be considered.

Keywords: next-generation sequencing (NGS), targeted gene panel sequencing, hypogammaglobulinemia, common variable immunodeficiency, genetic diagnosis, predominantly antibody deficiency, primary immunodeficiency

INTRODUCTION

Predominantly antibody deficiencies (PAD) are the most common form of inborn errors of immunity (IEI); they can present at any age and have a prevalence of approximately 1:10.000 (1). PAD comprise a diverse group of immune disorders characterized by increased susceptibility to multiple, recurrent and/or severe infections, impaired antibody production and poor response to vaccines (1). Among PAD, but also in this cohort, common variable immunodeficiency (CVID) is the most clinically important form of PAD, due to its relative prevalence (1:25.000 to 1:50.000) and the number of medical encounters (2). In addition, CVID is considered as a heterogeneous and intricate disorder since some individuals present almost with complete absence of all major immunoglobulin isotypes, while others have a reduction of one, two or three immunoglobulin isotypes, in variable combinations. Non-infectious complications, such as autoimmune conditions, lymphoid hyperplasia, granulomatous inflammation, and gastrointestinal inflammatory disease, have been observed in around 30 to 50% of CVID patients (3). However, the most common genetically diagnosed form of PAD is the X-linked agammaglobulinemia (XLA), which is caused by mutations in the Bruton's tyrosine kinase (*BTK*), and leads to a severe reduction of all serum immunoglobulin isotypes and absence of B cells (4). Additional forms of PAD may present with deficiency of one immunoglobulin isotype and with a milder clinical phenotype, as it is observed in patients with selective IgA deficiency, selective IgM deficiency or selective polysaccharide antibody deficiency (5). Up to date, about 40 different gene defects have been identified to primarily affect antibody production (6, 7); however, the genetic etiology is still unknown in up to 70-80% of patients (8). Noteworthy, the majority of CVID cases occur sporadically, and only 10 to 20% of the cases have a family history hinting towards a genetic origin. The latter is also observed in patients with selective IgA deficiency (9, 10).

The high percentage of unsolved cases might be due to limited genotype-phenotype correlations, polygenic traits, environmental factors, epigenetic causes and/or other genetic modifiers, as well as the lack of functional tests that could evaluate the deleteriousness of certain variants of uncertain significance (VUS).

In the last 10 years, the implementation of Next-Generation Sequencing (NGS) technologies have proven to be crucial in identifying the underlying genetic cause of many IEI (6, 11). Particularly, PAD-causing or PAD-associated mutations have been reported in more than 40 genes, according to the latest IEI classifications from the IUIS (6, 7). Many of these genes are not exclusively expressed in B cells, thereby leading to a more complex and variable clinical presentation in addition to hypogammaglobulinemia.

Depending on the underlying gene defect, patients might initially be diagnosed with PAD or CVID; however, as the disease progresses, additional viral and fungal infections, lung disease, autoimmune manifestations, autoinflammation, granulomatosis and/or malignancies can develop, complicate or dominate the clinical picture (12–14). Those manifestations indicate a more profound impairment and/or dysregulation of different components of the immune system. Consequently, some of the above-mentioned genetic defects are also found in patients diagnosed with combined immunodeficiency (CID) (e.g. mutations in *ICOS*, *LRBA*), with a CVID-like phenotype (e.g. *PLCG2*) (5), or with an immune dysregulation syndrome (e.g. *CTLA4*). These observations highlight the complexity of the pathomechanisms involved in PAD and CVID, since defects in B cell development, T-dependent and T-independent B cell activation, as well as in class switch recombination, have been shown to lead to hypogammaglobulinemia. These molecularly heterogeneous and clinically overlapping phenotypes challenge physicians when a solid diagnosis needs to be established. Therefore, genetic characterization in patients diagnosed with the different forms of PAD (including CVID, late-onset CID (loCID), or a CVID-like phenotype), is essential for an early molecular diagnosis. A genetic diagnosis may ensure a timely and appropriate treatment that prevents life-threatening infections and irreversible organ damage. Likewise, a molecular diagnosis helps with patient and family counselling and improves disease prognosis (1, 15).

In this study, we report our experience over the last 6 years employing targeted Next-Generation Sequencing (based on Agilent's HaloPlex or SureSelect designs and Illumina's MiSeq technologies) for a group of known disease-causing and other candidate genes in a cohort of 291 patients with PAD. Our purpose was to provide with a first-line genetic test to identify novel or known pathogenic variants in patients with PAD.

MATERIALS & METHODS

Patients

This study was conducted under the following ethics protocols: Vote no. 295/13 version 200149, Vote no. 60/18, Vote no. 290/13, and Vote no. 93/18 of the ethics committee of the University of

Freiburg, Germany. All patients and their parents (when patients were under 18 years of age) were consented to participate in our study according to local ethics guidelines. Whole blood samples from 291 patients, who presented - among other features - with recurrent infections and reduced immunoglobulin levels (only one of the major isotypes: IgA, IgG or IgM, or more than one, or IgG subclass deficiency), were collected in our outpatient clinic. Patients with low levels of immunoglobulins secondary to other diseases (e.g. kidney failure, hematologic neoplasms) or secondary to pharmacologic therapies (e.g. anti-epileptic or immune-suppressive drugs) were excluded from this study. In the 291 selected individuals no previous genetic testing had been performed, 284 were sporadic cases and seven were from three unrelated multiplex families.

In contrast to previous reports on PAD patients, no participant in our study was born to consanguineous parents. Familial segregation was studied when DNA samples from parents and siblings were available.

Panel Design

Between February 2014 and May 2020, various customized (Tier 1 or Tier 2) targeted panels were designed using Agilent's web-based SureDesign application. All panels included genes known to cause various types of inborn errors of immunity (IEI), but optionally also included additional putative candidate genes not previously associated with disease. The first panel (ID 3, **Supplementary Table 1**) initially comprised 27 genes, and over time, our IEI panel was updated regularly in order to include novel IEI-causing genes and to optimize sequencing depth and coverage, but still fitting the probe size of Agilent's Tier 1/2. In total 18 different panel designs were used in this study to screen the 291 individuals. Our latest and largest panel (ID 33) contained a total of 140 genes in 2020 (**Supplementary Table 1**).

DNA Extraction, Library Preparation and Sequencing

DNA extraction from peripheral blood samples treated with EDTA was performed according to our local protocol. Briefly, erythrocytes were lysed with our in-house RBC buffer. The remaining whole peripheral leukocytes were subjected to Qiagen Cell Lysis Solution (Qiagen, Hilden, Germany) for at least 24 hours at room temperature. Qiagen protein Precipitation Solution was used to precipitate the proteins. The DNA was then precipitated with isopropanol, washed with 70% ethanol and resuspended and stored in Qiagen DNA Hydration Solution. Concentration and purity were measured by fluorometric quantification (Qubit, Invitrogen/ThermoFisher Scientific, Langenselbold, Germany). Sample preparation, target enrichment and library preparation were performed using Agilent's HaloPlex or SureSelect enrichment system for Illumina sequencing following the manufacturer's instructions as detailed in Agilent's user manual (Illumina, Berlin, Germany; Agilent, Waldbronn, Germany). In brief, DNA samples were subjected to digestion by adding a restriction enzyme master mix prepared following the manufacturers protocol and an incubation step at 37°C. The digestion was validated by gel electrophoresis. Subsequently, the restriction fragments were hybridized to the HaloPlex or SureSelect probe capture library by addition of the Hybridization Master Mix as well as the indexing primer cassettes. After an incubation step, the hybridized DNA fragments were captured with a biotin-streptavidin system using HaloPlex magnetic beads. After a washing step, the circular fragments were closed through a ligation reaction, i.e. the ligation master mix was added and the solution was incubated at 55°C. Subsequently, the captured target libraries were amplified by PCR as suggested with the master mix prepared according to manufacturer's instructions. In a final step, the amplified target libraries were purified using AMPure XP beads and washed in 70% ethanol. Enrichment was validated on an Agilent TapeStation system. Then, samples were pooled in equimolar amount for multiplex sequencing on an Illumina MiSeq system following the manufacturer's protocol.

Bioinformatic Analysis and Variant Interpretation

Raw sequencing data (.fast files) were pre-processed according to GATK's best practices and included the following steps:.fastq file conversion into unmapped.bam files (PICARD tool: FastqToSam), tagging of illumine adapter sequences (PICARD tool: MarkIlluminaAdapters), conversion of tagged unmapped.bam file to.fastq file (PICARD tool: SamToFastq), sequence alignment to the human reference build hg38 (BWA MEM), identification of duplicated reads with PICARD tool: MarkDuplicates, and.bam file recalibration plus indel realignment. Variant calling was performed with GATK Haplotype caller, FreeBayes and SAMtools (16). The variants were then merged using custom BASH and R scripts, which included the unification of dinucleotide changes. Variant annotation was done using the Variant Effect Prediction (VEP) tool from ENSEMBL (https://www.ensembl.org/info/docs/tools/vep/index.html) and all results were imported into our internal database, which contains expert curated gene and variant information, and genetic (whole exome, targeted gene panel or single gene screening) and clinical data on more than 3,000 individuals. Short lists of candidate variants were generated from the database based on an (individual) frequency below 2% in our internal cohort or below 1% in the Genome Aggregation Database (gnomAD) - exomes and genomes - cohort, and a "high" or "moderate" predicted impact (**Supplementary Table 2**). However, published polymorphisms or risk alleles with a frequency up to 10% were also included. Variants were reported on the gene transcript with the highest predicted impact; however, the effect on additional gene transcripts were also available. The clinical relevance of all candidate variants was assessed following the updated guidelines (Sherloc) for the interpretation of sequence variants by the American College of Medical Genetics and Genomics–Association for Molecular Pathology (ACMG-AMP) (17). Most candidate variants were confirmed by assessing the aligned read pairs with the Integrated Genomics Viewer (IGV; Broad Institute) and, when required, validated by Sanger sequencing according to the standard protocols. In addition, familial segregation was studied when samples were available. Finally, as suggested by the guidelines, a deep literature review was

performed in order to confirm whether the genetic variations found in our study were previously reported, and if gene-disease correlations and/or experimental data demonstrating a detrimental effect had already been performed.

Variant Evaluation by Functional Assays

In order to evaluate the molecular and cellular consequence and prove the possible pathogenicity of some specific variants of interest, experimental tests measuring protein expression, phosphorylation, and/or function, were carried out in our laboratory. For this purpose, peripheral blood mononuclear cells (PBMCs) from affected patients and unaffected (travel or in-house) controls were used. In brief, PBMCs were isolated by density centrifugation and cultured with RPMI (Gibco/Thermo Fisher Scientific) medium supplemented with 10% fetal calf serum (Sigma-Aldrich/Merck, Darmstadt, Germany), 1 µg/ml penicillin and 1 µg/ml streptomycin (Invitrogen/Thermo Scientific). CTLA-4 transendocytosis and LRBA expression were assessed by flow cytometry as previously described (18–20). B-cell receptor (BCR) signaling assay (phosphorylation of Igα, SLP65 and BTK) and Ca^{2+} mobilization was determined as described before (21, 22). Data are shown after gating on naïve IgM+CD27-CD21+ or naive IgG-IgA-CD27-CD21+ B cells, respectively. Levels of Adenosine deaminase 1 or 2 (ADA and ADA2) were evaluated by measuring the specific enzyme activity in Michael S. Hershfield's lab at Duke University School of Medicine (USA) and at the Advanced Diagnostic Unit, University of Freiburg (Germany), respectively. *NFKB1* variants were analyzed as described previously (23).

RESULTS

Clinical and Genetic Characterization of 291 Patients With Suspected PAD

In this clinical and genetic study, we included a total of 291 patients who were seen at the outpatient clinic of the CCI in Freiburg and presented with hypogammaglobulinemia (reduction of at least one major immunoglobulin isotype) and a history of unusual or recurrent infections or other manifestations suggestive of altered immunity. There were 284 singleton cases and seven familial cases from three unrelated kindreds (F014: 2 sisters; F018: 3 cousins; F123: mother and daughter). Demographic features of this cohort are summarized in **Figure 1A**. The distribution age in this study was broad, with two main peaks in the second and fifth decade of life (**Figures 1A, B**). No history of consanguinity was reported. One patient deceased during the time of the study. Based on the immunoglobulin profile, reduction of all major isotypes (IgG, IgA and IgM) was reported in 50.1% of the patients, whereas 27.8% only had two out of the three isotypes reduced (IgG/IgA: 16.1%; IgM/IgA: 7.2% and IgG/IgM: 4.4%). Furthermore, 22% of the patients presented with either reduced IgG only (13.4%), IgM only (2.4%) or IgA only (6.1%). The age at first detection follows a normal distribution with two peaks at the second and fourth decade of life (**Figure 1C**).

Genetic Characterization Informative in up to 25% of the Investigated Cases

Sequencing of all 291 patients was performed between February 2014 and May 2020 and distributed in 45 runs, employing 18

FIGURE 1 | CCI Freiburg cohort: **(A)** Description of the cohort screened by targeted gene panel sequencing (TGP). **(B)** Age and gender distribution of the cohort at the time of the study. **(C)** Distribution of the cohort according to their reduced immunoglobulins profile and their age at first detection.

different custom targeted gene panel (TGP) designs, which were based either on Agilent's HaloPlex or SureSelect capture methods (**Supplementary Table 1** and **Supplementary Figure 1**). Distribution of the mean reading depth per sample varied across different runs and ranged from 300x to 4,200x for samples sequenced using HaloPlex, and from 50x to 1,700x for samples sequenced with SureSelect (**Supplementary Figure 1A**). Mean base pair coverage per sample was above 90% for most samples using HaloPlex, and uniformly above 98% for samples sequenced with SureSelect (**Supplementary Figure 1B**).

As expected, the number of variants identified in each sample positively correlated with the total number of base pairs and genes sequenced (**Supplementary Figure 2**). The total number of unfiltered variants per individual ranged from 50 to 1,600 (**Supplementary Figure 2A**). The number of rare variants (frequency below 1% in internal and/or external datasets) per individual ranged from 0 to 80 (**Supplementary Figure 2B**); and the number of candidate variants (rare variants with a "high" or "moderate" predicted impact) varied between 0 to 20 (**Supplementary Figure 2C**). Because 18 different panel designs were employed to sequence all samples, and because several samples were included in more than one experiment, the number of genes screened per individual (range: 20 to 204) varied across the cohort (**Supplementary Figure 2D**).

In this study, we screened more than 200 genes; however, some genes were sequenced only in a few patients, while others were sequenced in more than 285 subjects (**Figure 2A**). As expected, the genes in which we found several mutations were those that had been sequenced more often (**Supplementary Figures 2A, C, D**); except for genes such as *ADA*, which had been sequenced less than 150 subjects.

In 72 of the 291 patients included in the study, we were able to identify at least one genetic variant, which we considered pathogenic or likely pathogenic (**Table 1**) following the *Sherloc* guidelines (17). In these 72 patients, we identified 57 different genetic variants, allowing us to achieve a definite molecular diagnosis in 45 patients (15.5%) and a possible molecular diagnosis in 27 patients (9.3%) (**Figures 2B–D**). This accounts for a positive hit-rate of up to 24.7%. These 57 mutations comprised 28 missense, 13 frameshift, 9 nonsense and 7 splice-site variants in the following genes: *TNFRSF13B*, *CTLA4*, *NFKB1*, *STAT3*, *BTK*, *NFKB2*, *ADA2*, *LRBA*, *ADA* and *PIK3CD* (**Table 1**). Forty-Six of those 57 relevant variants had been previously associated with disease in the literature. Furthermore, we identified 11 novel variants, which we considered likely disease-causing mutations, in 10 patients. In addition to the 57 relevant variants, 24 additional genetic variants of uncertain clinical significance (VUS) were identified in 16 of the 72 definite/possible cases (**Table 2**). In the

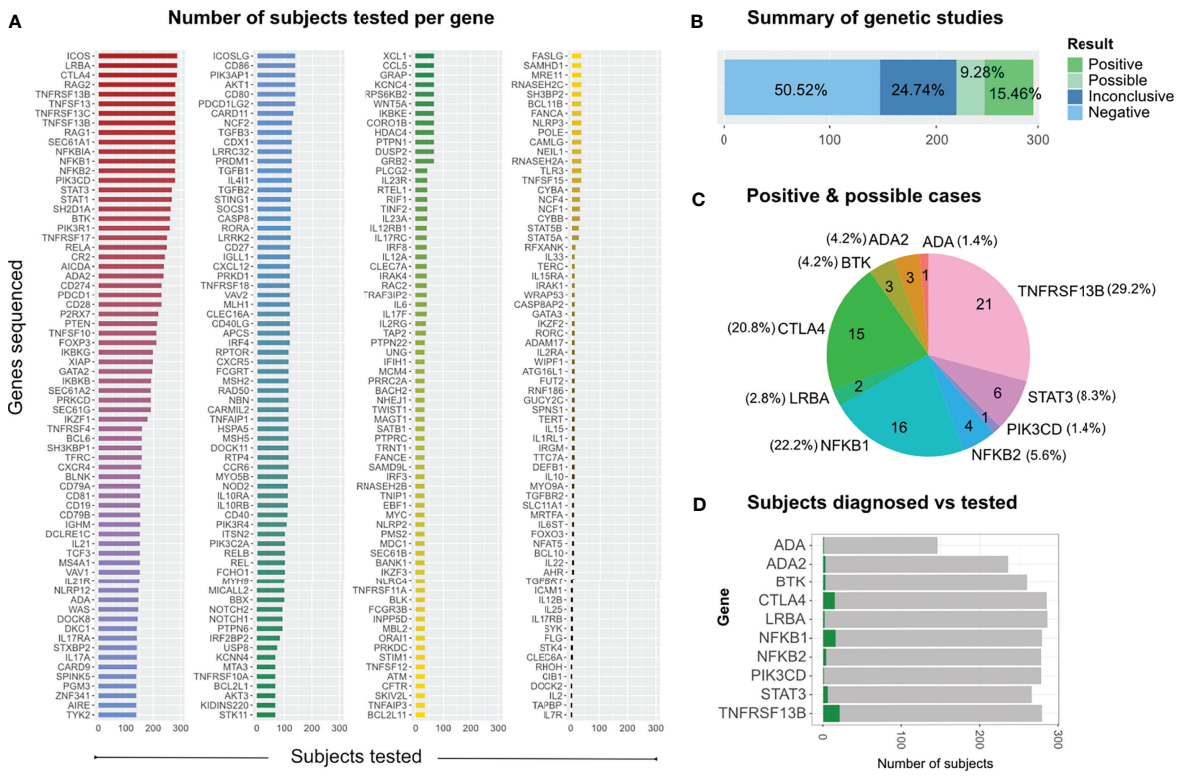

FIGURE 2 | Genetic screening and findings. **(A)** All genes sequenced across individual runs from 2014 to 2020 according to the number of subjects tested per gene. **(B)** Distribution of study results, which were classified as positive (definite), possible, inconclusive and negative (no relevant variants identified). **(C)** Distribution of relevant genetic findings. **(D)** Subjects diagnosed vs subjects tested per gene, limited to those 10 genes in which we found disease relevant mutations.

TABLE 1 | Detected mutations: Summary of detected disease-relevant variants by targeted panel sequencing (TGP).

Patient ID	Family ID	Gender (M/F)	Age at Diagnosis (years)	Molecular diagnosis	Gene	Chr. Location (GRCh38)	DNA change	Protein change	Reference sequence	Zygosity	Variant classification	Published (Variant/ patient)	Reference
P002	F002	M	40.81	CHAI	*CTLA4*	2:203871449	c.531_544del	p.Phe179Cysfs*29	ENST00000648405.2	Het	Pathogenic	Subject no. 87 or MM.II.1	(24, 25)
P005	F005	F	39.0	CVID12	*NFKB1*	4:102584821	c.1066+1G>C	predicted p.Phe310Ilefs*76 (if exon 11 is skipped)	ENST00000226574.9	Het	Pathogenic	Yes	(26)
P014	F014	F	56.0	CHAI	*CTLA4*	2:203868052	c.109+1G>T	NA	ENST00000648405.2	Het	Pathogenic	Family C	(18, 24, 25)
P015	F014	F	42.87	CHAI	*CTLA4*	2:203868052	c.109+1G>T	NA	ENST00000648405.2	Het	Pathogenic	Family C	(18, 24, 25)
P017	F017	M	25.0	CHAI	*CTLA4*	2:203870883	c.407C>T	p.Pro136Leu	ENST00000648405.2	Het	Pathogenic	Subject no. 17	(24, 25)
P018	F018	M	17.0	CHAI	*CTLA4*	2:203868047	c.105C>A	p.Cys35*	ENST00000648405.2	Het	Pathogenic	Family A	(18, 24)
P020	F018	F	19.0	CHAI	*CTLA4*	2:203868047	c.105C>A	p.Cys35*	ENST00000648405.2	Het	Pathogenic	Family A	(18, 24)
P021	F018	F	28.0	CHAI	*CTLA4*	2:203868047	c.105C>A	p.Cys35*	ENST00000648405.2	Het	Pathogenic	Family A	(18, 24)
P033	F033	F	12.0	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
					TNFRSF13B	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P039	F039	F	13.0	CVID12	*NFKB1*	4:102582898	c.872delA	p.Asn291Metfs*141	ENST00000226574.9	Het	Pathogenic	Yes	(26)
P048	F048	F	50.12	DADA2	*ADA2*	22:17209606	c.68_71del	p.Phe23Serfs*7	ENST00000399837.8	Hom	Pathogenic	No	–
P053	F053	M	35	CHAI	*CTLA4*	2:203870636	c.165_190dup	p.Gly64Alafs*17	ENST00000648405.2	Het	Pathogenic	No	–
P056	F056	M	40.61	CHAI	*CTLA4*	2:203870832	c.356T>G	p.Leu119Arg	ENST00000648405.2	Het	Pathogenic	Yes[§] (variant)	(24)
P059	F059	M	55.53	CVID8	*LRBA*	4:150350017	c.7370C>G	p.Ser2457*	ENST00000357115.8	Het	Pathogenic	No	–
					LRBA	4:150828208	c.5143C>T	p.Gln1715*	ENST00000357115.8	Het	Pathogenic	No	–
P064	F064	M	23.70	CVID2	*TNFRSF13B*	17:16940442	c.515G>A	p.Cys172Tyr	ENST00000261652.6	Het	Risk allele	Yes (variant)	(27, 29, 30, 33)
P069	F069	F	37.28	CVID12	*NFKB1*	4:102576988	c.520_521insCTGA	p.Leu176*	ENST00000226574.9	Het	Pathogenic	Yes (variant)	(26)
P073	F073	M	21.0	CVID12	*NFKB1*	4:102578938	c.634_656dup	p.Phe220Trpfs*40	ENST00000226574.9	Het	Pathogenic	No	-
P093	F093	F	18.0	CVID2	*TNFRSF13B*	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P098	F098	M	27.0	CHAI	*CTLA4*	2:203870699	c.223C>T	p.Arg75Trp	ENST00000648405.2	Het	Pathogenic	Subject no. 83	(18)
P101	F101	F	73.0	IMAD1	*STAT3*	17:42322384	c.1999G>T	p.Val667Leu	ENST00000264657.9	Het	Likely pathogenic	Yes[+] (variant)	(34)
P103	F103	F	21.22	CVID2	*TNFRSF13B*	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P108	F108	M	50.0	CVID12	*NFKB1*	4:102613501	c.2671delG	p.Ala891Glnfs*6	ENST00000226574.9	Het	Pathogenic	Yes (variant)	(35)
P122	F122	F	25.0	CHAI	*CTLA4*	2:203870802	c.326G>A	p.Gly109Glu	ENST00000648405.2	Het	Likely Pathogenic	Yes[§] Subject 127	(24)
P123	F123	F	52.0	CVID10	*NFKB2*	10:102402138	c.2557C>T	p.Arg853*	ENST00000369966.8	Het	Pathogenic	Pt#22 of	Fam1404

(Continued)

TABLE 1 | Continued

Patient ID	Family ID	Gender (M/F)	Age at Diagnosis (years)	Molecular diagnosis	Gene	Chr. Location (GRCh38)	DNA change	Protein change	Reference sequence	Zygosity	Variant classification	Published (Variant/ patient)	Reference
(36)													
P124 (36)	F123	F	24.48	CVID10	*NFKB2*	10:102402138	c.2557C>T	p.Arg853*	ENST00000369966.8	Het	Pathogenic	Pt#23 of	Fam1404
P125	F125	M	36.75	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P134	F134	M	58.0	CVID12	*NFKB1*	4:102566997	c.269A>C	p.Tyr90Ser	ENST00000226574.9	Het	Likely Pathogenic	Yes (variant)	(26)
P135	F135	M	50.41	CVID2	*TNFRSF13B*	17:16940378	c.579C>A	p.Cys193*	ENST00000261652.6	Het	Risk allele	Yes (variant)	(27)
P138	F138	M	51.94	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P141	F141	F	51.13	DADA2	*ADA2*	22:17209538	c.140G>C	p.Gly47Ala	ENST00000399837.8	Het	pathogenic	Patient 4	(37–39)
P143	F143	M	29	CVID10	*NFKB2*	10:102402284	c.2611C>T	p.Gln871*	ENST00000369966.8	Het	Pathogenic	Yes (variant)	(40)
P153	F153	M	59.0	CVID12	*NFKB1*	4:102576937	c.469C>T	p.Arg157*	ENST00000226574.9	Het	Pathogenic	Yes	(26)
P154	F154	M	32.0	IMAD1	*STAT3*	17:42333990	c.857A>C	p.Glu286Ala	ENST00000264657.9	Het	Pathogenic	Yes (variant)	(41)
P156	F156	F	35.0	CVID12	*NFKB1*	4:102580641	c.835+2T>G	p.Lys244_Asp279delinsAsn	ENST00000226574.9	Het	Pathogenic	Yes	(26, 42)
P160	F160	F	53.0	CVID12	*NFKB1*	4:102596201	c.1365del	p.Val456*	ENST00000226574.9	Het	Pathogenic	Yes	(26)
P163	F163	M	49.0	CVID12	*NFKB1*	4:102612609	c.2592+3A>G	Predicted p.Asp808Leufs*22 if exon 22 is skipped; retaining intron 22 predicts p.Ser866_Lys968delins10	ENST00000226574.9	Het	Pathogenic	No	–
P170	F170	M	11.58	IMAD1	*STAT3*	17:42333984	c.863A>C	p.Gln288Pro	ENST00000264657.9	Het	Pathogenic	Yes (variant)	(41)
P173	F173	F	11.0	CVID8	*LRBA*	4:150852870	c.2836_2839del	p.Glu946*	ENST00000357115.8	Het	Pathogenic	Yes (variant)	(43)
					LRBA	4:150908407	c.1420C>T	p.Gln474*	ENST00000357115.9	Het	Pathogenic	Yes (variant)	(43)
P182	F182	M	40.30	IMAD1	*STAT3*	17:42316899	c.2147C>T	p.Thr716Met	ENST00000264657.9	Het	Pathogenic	Yes (variant)	(44, 45)
P188	F188	M	4.32	CVID12	*NFKB1*	4:102578955	c.646A>G	p.Met216Val	ENST00000226574.9	Het	Pathogenic	Yes	(26)
P192	F192	F	53.0	CVID12	*NFKB1*	4:102580641	c.835+2T>G	p.Lys244_Asp279delinsAsn	ENST00000226574.9	Het	Pathogenic	Yes	(26, 42)
P196	F196	F	9	IMAD1	*STAT3*	17:42334008	c.839A>C	p.Gln280Pro	ENST00000264657.9	Het	Pathogenic	Yes (variant)	(41)
P198	F198	M	46.0	DADA2	*ADA2*	22:17182620	c.1223G>A	p.Cys408Tyr	ENST00000399837.8	Het	Pathogenic	Patient 2	(37)
					ADA2	22:17207070	c.542+1G>A	NA	ENST00000399837.8	Het	Pathogenic	Patient 2	(37)
P206	F206	M	52.0	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
					TNFRSF13B	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)

(Continued)

TABLE 1 | Continued

Patient ID	Family ID	Gender (M/F)	Age at Diagnosis (years)	Molecular diagnosis	Gene	Chr. Location (GRCh38)	DNA change	Protein change	Reference sequence	Zygosity	Variant classification	Published (Variant/ patient)	Reference
P212	F212	M	55.0	CVID2	*TNFRSF13B*	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P215	F215	M	49.89	XLA1	*BTK*	X:101354640	c.1723G>T	p.Gly575Cys	ENST00000621635.4	Hem	Likely Pathogenic	No	–
P217	F217	F	16.0	CHAI	*CTLA4*	2:203870627	c.151C>T	p.Arg51*	ENST00000648405.2	Het	Pathogenic	Subject no. 128[§]	(24)
P219	F219	M	23.42	CVID12	*NFKB1*	4:102578950	c.641G>A	p.Arg214Gln	ENST00000226574.9	Het	Pathogenic	Yes	(26)
P220	F220	M	9.0	CVID10	*NFKB2*	10:102402268	c.2596_2597del	p.Ser866Cysfs*19	ENST00000369966.8	Het	Pathogenic	Pt#49 (Fam846)	(26, 36)
P221	F221	F	50.25	DADA1	*ADA*	20:44621082	c.911T>G	p.Leu304Arg	ENST00000372874.9	Het	Pathogenic	Yes (variant)	(46)
					ADA	20:44621103	c.890C>T	p.Pro297Leu	ENST00000372874.9	Het	Pathogenic	No	–
P236	F236	M	47.0	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
					TNFRSF13B	17:16939723	c.706G>T	p.Glu236*	ENST00000261652.6	Het	Risk allele	Yes (variant)	(27)
P250	F250	M	48.43	CVID2	*TNFRSF13B*	17:16948978	c.204dup	p.Leu69Thrfs*12	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P251	F251	M	17.0	CVID2	*TNFRSF13B*	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P258	F258	M	31.10	CHAI	*CTLA4*	2:203870886	c.410C>T	p.Pro137Leu	ENST00000648405.2	Het	Pathogenic	Subject no.97	(25)
P259	F259	M	15.0	CVID12	*NFKB1*	4:102584765	c.1012delT	p.Ser338Leufs*94	ENST00000226574.9	Het	Pathogenic	Yes	(26, 47)
P260	F260	M	22.72	CHAI	*CTLA4*	2:203870909	c.433_434insACGG	p.Thr147Argfs*8	ENST00000648405.2	Het	Pathogenic	No	–
P264	F264	F	23.0	IMAD1	*STAT3*	17:42346635	c.207C>A	p.Ser69Arg	ENST00000264657.9	Het	Likely Pathogenic	No	–
P265	F265	F	44.0	CVID2	*TNFRSF13B*	17:16948923	c.260T>A	p.Ile87Asn	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 30)
P271	F271	F	30.0	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P274	F274	F	69.68	CVID12	*NFKB1*	4:102582898	c.872delA	p.Asn291Metfs*141	ENST00000226574.9	Het	Pathogenic	Yes (variant)	(26)
P281	F281	M	19.67	XLA-1	*BTK*	X:101375203	c.184C>T	p.Arg62Cys	ENST00000621635.4	Hem	Pathogenic	Yes (variant)	(48, 49)
P295	F295	M	40.30	XLA-1	*BTK*	X:101360688	c.757del	p.Val253Leufs*10	ENST00000621635.4	Hem	Pathogenic	Yes (variant)	(50)
P301	F301	F	53.2	CVID2	*TNFRSF13B*	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P306	F306	F	58.0	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P311	F311	F	34.0	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P341	F341	F	49.78	CHAI	*CTLA4*	2:203870594	c.118G>A	p.Val40Met	ENST00000648405.2	Het	Likely Pathogenic	Yes[§] (variant)	(51, 52)

(Continued)

TABLE 1 | Continued

Patient ID	Family ID	Gender (M/F)	Age at Diagnosis (years)	Molecular diagnosis	Gene	Chr. Location (GRCh38)	DNA change	Protein change	Reference sequence	Zygosity	Variant classification	Published (Variant/patient)	Reference
P397	F397	F	17	IMD14A	*PIK3CD*	1:9726972	c.3061G>A	p.Glu1021Lys	ENST00000377346.9	Het	Pathogenic	Yes (variant)	(53–55)

Patient ID	Family ID	Gender (M/F)	Age at Diagnosis (years)	Molecular diagnosis	Gene	Chr. Location (GRCh38)	Coding change	Protein change	Transcript identifier	Zygosity	Variant classification	Published (Variant/patient)	Reference
P398	F398	M	18	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P404	F404	M	56.1	CVID2	*TNFRSF13B*	17:16940415	c.542C>A	p.Ala181Glu	ENST00000261652.6	Het	Risk allele	Yes (variant)	(29–32)
P405	F405	M	29	CVID2	*TNFRSF13B*	17:16933171	c.451C>T	p.Arg151*	ENST00000579315.5	Het	Likely risk allele	No	–
P406	F406	F	60	CVID2	*TNFRSF13B*	17:16948873	c.310T>C	p.Cys104Arg	ENST00000261652.6	Het	Risk allele	Yes (variant)	(9, 27, 28)
P413	F413	M	15.8	CVID12	*NFKB1*	4:102537867	c.169C>T	p.Arg57Cys	ENST00000226574.9	Het	Pathogenic	Yes	(26)

M, Male; F, Female.

§variant published in patients with clinical manifestations of CTLA4 or CVID-like but not functionally tested.

†published as somatic mutation in a patient with peripheral T-cell lymphoma (PTCL) (34).

Hom, homozygous; het, heterozygous; hemi, hemizygous; CHAI, CTLA4 haploinsuficiency [OMIM: 616100]; CVID2, TACI deficiency [OMIM: 240500]; CVID8, LRBA deficiency [OMIM: 614700]; CVID10,NFKB2 deficiency [OMIM: 615577]; CVID12, NFKB1 haploinsufficiency [OMIM, 616576]; IMAD1, Infantile-Onset Multisystem Autoimmune Disease 1 [OMIM: 615952]; DADA1, Adenosine deaminase 1 deficiency [OMIM: 608958]; DADA2, Adenosine deaminase 2 deficiency [OMIM: 607575]; XLA1, X-linked agammaglobulinemia [OMIM: 300755]; IMD14A, Immunodeficiency 14A [OMIM: 615513].

** represents the premature stop/termination translation codon.*

remaining 219 patients, we only detected one or more VUS plus benign or likely benign variants, which were not sufficient to obtain a clear and conclusive molecular diagnosis and thus classified as 'inconclusive' (72 cases, 24.7%); or we detected benign variants or non-benign variants that did not fit with the mode of inheritance, and thereby were not considered disease-relevant and classified as 'negative' (147 cases, 50.5%) (**Figure 2B**).

The majority of our 72 definite/possible patients were found to carry mutations in genes associated with autosomal dominant (AD) conditions, whereas a minority of patients carried disease-relevant mutations in genes associated with autosomal recessive (AR) or X-linked inheritance (XLR) (**Supplementary Figure 3B**). The mean age at first detection of hypogammaglobulinemia was 28.9 years for the positive/possible cases, and 32.7 years for the unsolved cases.

Clinical Characteristics of the 72 Definite and Possible Cases

As mentioned above, the detection of low antibody levels - of any of the three major immunoglobulin isotypes - and a history of unusual infections (or other indication of altered immunity) were the common clinical manifestations of the entire cohort of 291 subjects. Among the 72 definite/possible cases, unusual infections (bacterial [29.2%], fungal [9.7%], viral [44.4%] or unclassified [15.3%]) were observed in 95.8% of patients. Of these, 84.7% suffered from recurrent infections of the respiratory tract, and 73.6% individuals developed lung disease, including interstitial lung disease (ILD, 37.5%), bronchiectasis (27.8%) and chronic obstructive pulmonary disease (COPD, 1.4%). Abnormal lymphocyte proliferation and gastrointestinal manifestations were found in 66.7% and 48.6% of the 72 patients, respectively. Autoimmune conditions were observed in 45.8%, and skin abnormalities (including warts) in 37.5% of individuals. Finally, 40.3% of the molecular diagnosed patients suffered from different allergies and 11.1% of patients developed lymphoma (**Figure 3**).

Variants in *TNFRSF13B* (TACI) Are the Most Frequent Sequence Changes in the Freiburg Cohort

Variants in *TNFRSF13B* were the most prevalent in our cohort, observed in 28 (9.6%) of the 291 patients in our cohort, comparing to 2.8% in controls (60,146 individuals) reported in the gnomAD database v2.1 or 2.8% in our internal control cohort (84 individuals with normal immunoglobulin levels) (**Figure 4A**). These observations are in line with previous reports on PAD or CVID cohorts (8, 56, 57).

Specifically, 12 distinct rare variants (AF < 0.01 in gnomAD exomes/genomes) in *TNFRSF13B*, were identified in 28 (9.6%) patients (**Table 3** and **Figure 4B**). Eleven out of these 12 variants were considered in this study as risk alleles and not as disease-causing since they were also found – yet at a lower frequency – in internal and external (gnomAD) controls. The remaining variant was not considered as a risk allele, since it has been reported as a polymorphism occurring at similar frequencies in affected individuals and controls (9, 58, 60). As expected, the p.Cys104Arg and p.Ala181Glu variants were found in more than 3.5% of patients, whereas the 10 additional variants were found in less

TABLE 2 | Detected variants of uncertain significance in the 72 definite/possible cases.

Patient ID	Gene	Chr.Location (GRCh38)	Coding change	Protein change	Transcript identifier	Zygosity
P018	*F5*	1:169549811	c.1601G>A	p.Arg534Gln	ENST00000367797.8	Het
P033	*NOD2*	16:50729867	c.3019dup	p.Leu1007Profs*2	ENST00000300589.6	Het
P056	*NOD2*	16:50729867	c.3019dup	p.Leu1007Profs*2	ENST00000300589.6	Het
P064	*MYH9*	22:36285884	c.5131G>A	p.Ala1711Thr	ENST00000216181.10	Het
P101	*PIK3C2A*	11:17169343	c.399T>G	p.Phe133Leu	ENST00000265970.11	Het
	NOD2	16:50722629	c.2722G>C	p.Gly908Arg	ENST00000300589.6	Het
	PIK3C2A	11:17089793	c.5006A>G	p.Asn1669Ser	ENST00000265970.11	Het
P125	*NOD2*	16:50712175	c.2264C>T	p.Ala755Val	ENST00000300589.6	Het
	P2RX7	12:121162435	c.448G>A	p.Gly150Arg	ENST00000328963.10	Het
P138	*RELA*	11:65659712	c.513G>T	p.Arg171Ser	ENST00000406246.8	Het
P143	*PRKD1*	14:29927423	c.90_91ins6	p.Gly30_Ser31insAspPro	ENST00000415220.6	Het
	NOTCH2NLC	1:149390805	c.18_19insAGG	p.Gly6_Gly7insArg	ENST00000650865.1	Het
P154	*STAT1*	2:191009873	c.128+3A>G	–	ENST00000361099.7	Het
P182	*WAS*	X:48686945	c.724A>T	p.Ser242Cys	ENST00000376701.4	Hem
P212	*TNFRSF13C*	22:41925447	c.475C>T	p.His159Tyr	ENST00000291232.4	Het
P251	*GATA2*	3:12848131	c.1145delinsG	p.Val382Gly	ENST00000341105.7	Het
P274	*NOD2*	16:50712018	c.2107C>T	p.Arg703Cys	ENST00000300589.6	Het
P301	*IFIH1*	2:162279995	c.1641+1G>C	–	ENST00000263642.2	Het
	CARD11	7:2913449	c.2857G>A	p.Glu953Lys	ENST00000396946.8	Het
	NLRP12	19:53810880	c.779C>T	p.Thr260Met	ENST00000391773.5	Het
	IRF3	19:49663457	c.223A>G	p.Thr75Ala	ENST00000601291.5	Het
	NFKB1	4:102533876	c.150A>C	p.Gln50His	ENST00000226574.9	Het
	NFKB1	4:102533882	c.156A>C	p.Lys52Asn	ENST00000226574.9	Het
P311	*NOD2*	16:50712175	c.2264C>T	p.Ala755Val	ENST00000300589.6	Het
P405	*STAT3*	17:42329763	c.1123G>A	p.Val375Ile	ENST00000264657.9	Het

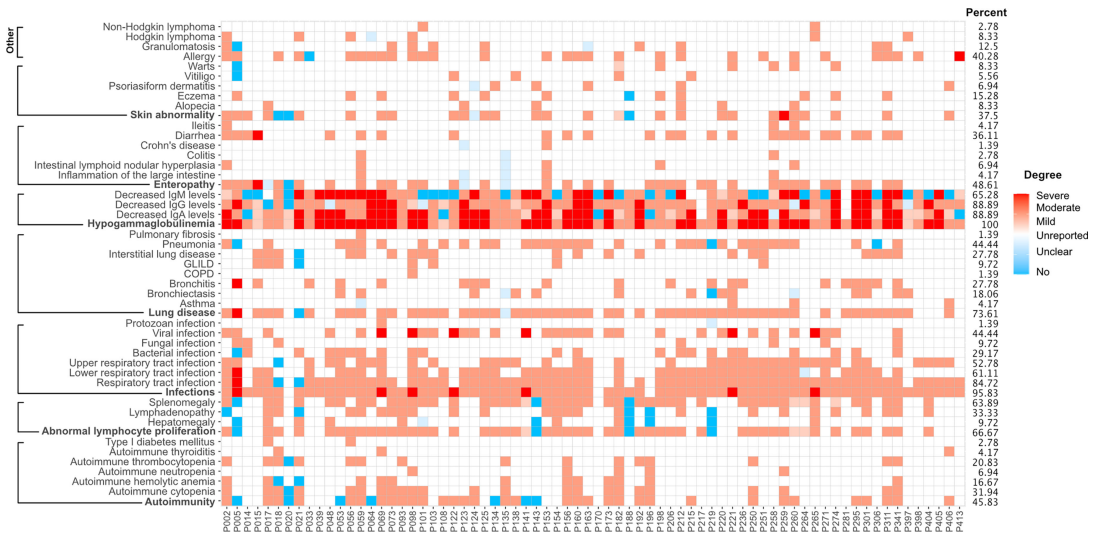

FIGURE 3 | Clinical manifestations of 72 patients with a definite or possible molecular diagnosis. Presence and degree of manifestations are color-coded. Blue squares, absence; light blue squares, unclear; white squares, unreported finding; light red, mild manifestation; medium red, moderate or typical presentation; dark red squares, severe manifestation.

than 1% of patients in this study (**Figure 4B**). Fifteen of these 28 patients carried a known monoallelic missense variant (p.Ala181Glu, p.Cys104Arg, p.Cys172Tyr, p.Ile87Asn [ENST00000261652.6]), whereas three patients carried novel monoallelic missense VUS (p.Ser123Thr, p.Asp191Asn, p.Asp85Glu). However, four patients (P002, P018, P219, P264) not only carried heterozygous missense variants in TACI (p.Arg72His, p.Ala181Glu, p.Cys104Arg), but also deleterious variants in *CTLA4* or a possibly deleterious variant in *STAT3* or *NFKB1*. Furthermore, one patient (P250) was heterozygous for a known duplication (c.204dupT) in *TNFRSF13B*, leading to a frameshift and a premature termination of translation (p.Leu69Thrfs*12), and two patients (P135 and P405) were heterozygous for the known p.Cys193* and the novel p.Arg151*

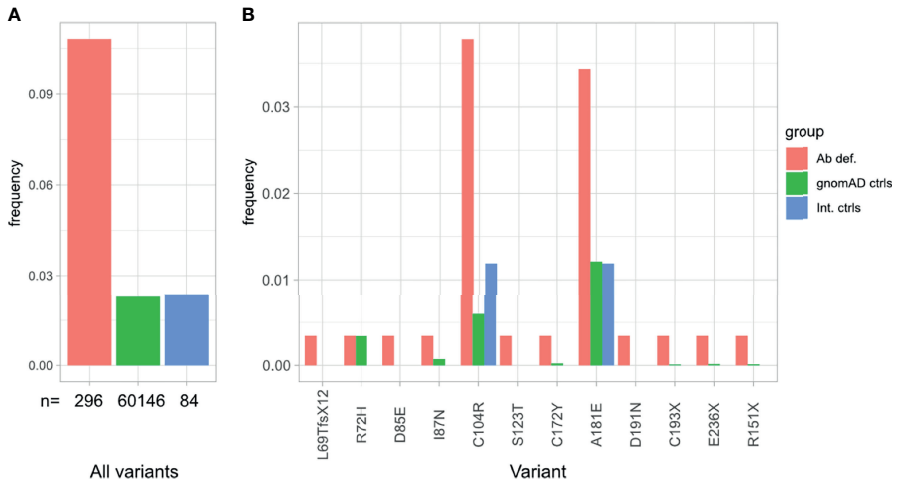

FIGURE 4 | Overrepresentation of *TNFRSF13B* variants in patients with antibody deficiency compared to internal and external controls. **(A)** Frequency of *TNFRSF13B* variant carriers in our cohort in comparison to controls reported in gnomAD and internal controls from our database. **(B)** Frequency and distribution of the variants found in *TNFRSF13B* in this study, compared to their frequency in control populations.

TABLE 3 | Detected mutations in *TNFRSF13B*: rare variants with an AF < 0.01 in gnomAD exomes/genomes found in 28 of 291 patients.

Patient ID	Zygosity	Chr.location	Coding change	Protein change	Variant classification	AF gnomAD controls v2.1	Published
P002	Het	17:16948968-C-T	c.215G>A	p.Arg72His	Polymorphism	0.001713	(27, 29, 58)
P013	Het	17:16948815-C-G	c.368G>C	p.Ser123Thr	Uncertain	–	No
P018	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P033	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P064	Het	17:16940442-C-T	c.515G>A	p.Cys172Tyr	Risk allele	0.000117	(27, 29, 30, 33)
P086	Het	17:16940386-C-T	c.571G>A	p.Asp191Asn	Uncertain	–	No
P093	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P103	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P125	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P135	Het	17:16940378-G-T	c.579C>A	p.Cys193*	Risk allele	0.000046	(27)
P138	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P206	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P212	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P219	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P236	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
	Het	17:16939723-C-A	c.706G>T	p.Glu236*	Risk allele	0.000075	(27)
P250	Het	17:16948978-G-GT	c.204dup	p.Leu69Thrfs*12	Risk allele	0.000233	(9, 27, 28)
P251	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P264	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P265	Het	17:16948923-A-T	c.260T>A	p.Ile87Asn	Risk allele	0.000366	(9, 27, 30, 59)
P271	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P301	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P306	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P311	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P316	Het	17:16948928-G-C	c.255C>G	p.Asp85Glu	Uncertain	–	No
P398	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)
P404	Het	17:16940415-G-T	c.542C>A	p.Ala181Glu	Risk allele	0.006136	(29–32)
P405	Het	17:16933171-G-A	c.451C>T	p.Arg151*	Likely risk allele	–	No
P406	Het	17:16948873-A-G	c.310T>C	p.Cys104Arg	Risk allele	0.002993	(9, 27, 28)

** represents the premature stop/termination translation codon.*

(ENST00000579315.5) nonsense mutations in *TNFRSF13B*, respectively. The remaining three individuals had biallelic *TNFRSF13B* mutations: two unrelated patients (P206, P033) carried the combination of p.Cys104Arg and p.Ala181Glu, and one patient (P236) had the p.Cys104Arg mutation together with a nonsense p.Glu236* mutation. There was no noticeable

difference in the clinical presentation of the heterozygous *versus* the compound heterozygous *TNFRSF13B* variant carriers. In summary, 21 of the 28 patients with variants in *TNFRSF13B* were classified as having a "possible" genetic diagnosis (**Figure 1**), whereas the three heterozygous patients with the three novel mutations were classified as "inconclusive". The four patients with co-existing mutations in *CTLA4*, *NFKB1* and *STAT3* were classified as CTLA4, NFKB1 or STAT3 patients, respectively, but not within the group of patients carrying variants in *TNFRSF13B*.

Mutations in *NFKB1* and *NFKB2* Collectively Account for 27.8% of the Solved Cases in Our Cohort

We found that the clinical phenotype of many of our patients could be genetically explained by monoallelic *NFKB1* mutations and, less commonly, by *NFKB2* mutations (**Figure 2C** and **Table 1**). A total of 16 patients carried relevant mutations in *NFKB1*. Fourteen of whom were found to have severe N-terminal truncating mutations. These N-terminal truncating mutations lead to haploinsufficiency of both, the p105 precursor protein (encoded by *NFKB1*) and the mature p50 (which is generated by proteasome-mediated removal of the C-terminal half of p105). Patient P153 had the known stop-gain mutation p.Arg157* (61, 62). Patients P039 and P274 (from unrelated families) both carried the single base pair deletion (c.872delA; p.Asn291Metfs*141). Patient P259 had the c.1012delT; p.Ser338Leufs*94 mutation, and P069 carried a 4-base pair insertion (c.520_521insCTGA; p.Leu176*). All these individuals were reported in 2020 as part of the cohort studied by Lorenzini and colleagues (26). Patient P073 had not been previously reported and had a novel 23bp duplication (c.634_656dup; p.Phe220Trpfs*40). This mutation is also predicted to disrupt both, the precursor p105 and the mature form p50 of NF-κB1. In analogy to other well-known severely truncating mutations (63), we consider this newly identified variant as pathogenic, although we have not explicitly confirmed its deleterious effect. Patients P005, P156 and P192 (all unrelated) carried splice-altering mutations: The splice-donor change c.1066+1G>C (which was found in P005) results in a shift of the reading frame and a premature termination of translation (p.Phe310Ilefs*76); however, other splice defects are also conceivable. The change c.835+2T>G, which is found in P156 and P192, leads to in-frame skipping of exon 9 and causes an internal deletion of 36 amino acids and insertion of an asparagine residue due to the fusion of exon 8 and 10 (p.LysK244_Asp279delinsAsn) (42). These patients were also included in the Lorenzini et al. cohort (26, 42).

In addition, we identified four patients carrying four missense variants: P134 (c.269A>C; p.Tyr90Ser), P188 (c.646A>G; p.Met216Val), P413 (c.169C>T; p.Arg57Cys), and patient P219 (c.641G>A; p.Arg214Gln). The latter patient additionally carried the known p.Cys104Arg mutation in *TNFRSF13B*. Although functional *in vitro* testing, as previously described (23), indicated that none of these four variants cause a detrimental protein loss, we could not exclude a hypomorphic reduction of protein function (data not shown). Using reporter assays, a recent study demonstrated a loss-of-function for p.Arg57Cys, whereas a functional defect associated with p.Tyr90Ser, p.Arg214Gln, and p.Met216Val, remained obscure (63). P134 presented with splenomegaly, pneumonias and psoriatic dermatitis. P219 suffered from recurrent respiratory tract infections and gastrointestinal manifestations. P413 presented with severe allergy. Unfortunately, detailed clinical data from patient P188 were not available (**Figure 3**). Furthermore, patient P160, who was also included in the NFKB1 cohort reported previously (26), carried a single-nucleotide deletion (c.1365delT; p.Val456*) in the central part of *NFKB1*. This particular mutation predicts skipping the precursor p105 stage and the immediate expression of p50-like mutant proteins (64, 65). P160 presented with recurrent viral and bacterial infections, autoimmune hemolytic anemia (AIHA), abnormal lymphoproliferation, and allergy. Finally, we detected two subjects carrying truncating mutations that affect the C-terminal portion of *NFKB1*: Patient P108 harbors a single-nucleotide deletion (c.2671delG; p.Ala891Glnfs*6), which is predicted to alter the amino acid sequence of the death domain (DD) of the p105 precursor (35). Patient P163 was found to have a putative splice donor mutation (c.2592+3A>G), predicting the expression of an abnormal precursor protein (p.Asp808Leufs*22 if the variant leads to skipping of exon 22; or p.Ser866_Lys968delins10 if intron 22 is retained). To date, the specific defects of the C-terminally truncated p105 proteins remain unknown.

With regard to *NFKB2*, we identified four patients carrying disease-relevant mutations: P123 and P124 (mother and her daughter), who were previously described by Klemann et al. in 2019 as Pt#22 and Pt#23 of Fam1404 (36), carry the most-frequent dominant-negative nonsense mutation (c.2557C>T; p.Arg853*) in *NFKB2* (66, 67). Patient P143 was heterozygous for the previously published stop-gain mutation: c.2611C>T; p.Gln871* (36, 40) and suffered from recurrent upper and lower respiratory tract infections, alopecia, psoriasiform dermatitis, vitamin D deficiency, and osteoporosis. Subject P220, who was also reported in the above study as Pt#49 (Fam846) (36), carries a *de novo* heterozygous deletion (c.2596_2597delAG; p.Ser866Cysfs*19) (**Table 1**).

Fifteen of the 291 Investigated Patients Have Mutations in *CTLA4*

Among the 291 investigated patients, 15 patients from 12 unrelated families were found to carry relevant mutations in *CTLA4*. The mutations in *CTLA4* included one single-nucleotide splice-site mutation (c.109+1G>T), which is known to affect the mRNA splicing (18). This mutation was found in two affected sisters (patients P014 and P015 from family F014). These women were initially reported in 2014 by Schubert et al. as Family C (18). Three individuals were found to carry known stop-gain mutations in residues located in the ligand-binding domain of the protein: Patient P018 from family F018, who also carried the known variant p.Ala181Glu in *TNFRSF13B*, had the p.Cys35* nonsense variant in *CTLA4* (18). This mutation is also present in two of his cousins (P020, P021). This family had also been reported before (Family A) by Schubert et al. in 2014 (18). Patient P217 had the p.Arg51* mutation (she was previously

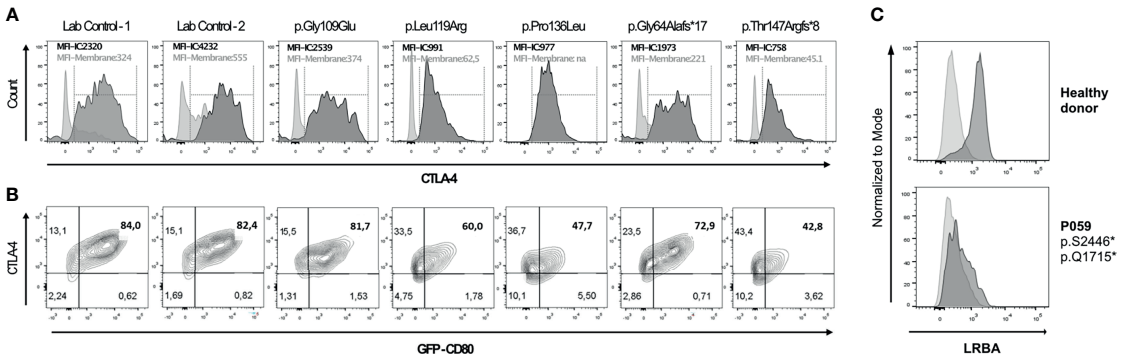

FIGURE 5 | Functional assessment of novel genetic variants in *CTLA4* and LRBA individuals by flow cytometry. **(A)** Histogram overlays show CTLA-4 expression on the surface (light grey) and total intracellular CTLA-4 (dark grey) in activated CD4+ FOXP3+ (Treg) cells. **(B)** Ligand binding uptake of GFP-CD80 by stimulated primary CD4+FOXP3 primary cells of two controls and four patients. Flow cytometry plots depict the percentage of GFP-CD80. **(C)** Histogram overlays show isotype (light grey) and LRBA expression (dark grey) in peripheral blood mononuclear cells stimulated with phytohaemagglutinin (PHA) for 72h in a healthy donor (HD) and in patient 059 (P059).

reported as subject no. 128 by Schwab et al. in 2018) (24). Three individuals were found to carry frameshift mutations: Patient P002, who was previously reported as subject no. 87 (24) or MM.II.1 (25), harbored a 14-base-pair deletion (c.530_543del; p.Phe179Cysfs*29). Patients P260 and P053 carried a novel 4-base pair insertion (c.433_434insACGG; p.Thr147Argfs*8), and a novel 35-base pair duplication (c.165_190dup; p.Gly64Alafs*17, respectively. Functional evaluation showed low levels of intracellular CTLA-4 expression and a reduced percentage of CD80-ligand uptake for the p.Thr147Argfs*8 and the p.Gly64Alafs*17 mutants (**Figure 5**). P260 and P053 suffered from recurrent and severe respiratory tract infections, which ultimately led to the development of bronchiectasis. However, P260 had additional clinical manifestations such as enteropathy, nodular lymphoid hyperplasia, ileitis, and skin abnormalities. Six patients were found to bear missense mutations, four of whom had been previously reported: P098 [subject no. 83 (24)] [p.Arg75Trp], P258 [subject no. 97 (24)] [p.Pro137Leu], P017 [subject no. 17 (24)] [p.Pro136Leu], and P122 [subject 127 (24)] [p.Gly109Glu]. Two of them, however, have previously not been reported: P341 [p.Val40Met] and P056 [p.Leu119Arg]. P341 presented with autoimmune cytopenia, recurrent lower respiratory tract infections, lymphoproliferative features and enteropathy. Patient P056 presented with type I diabetes, autoimmune thrombocytopenia (ITP), lymphadenopathy, ILD, eczema, and Hodgkin lymphoma. Nevertheless, four out of these seven missense variants had not been experimentally confirmed to functionally affect the biology of CTLA-4. Further work-up revealed reduced intracellular CTLA-4 expression for patients' cells carrying the variants p.Pro136Leu (68) and p.Leu119Arg; whereas intracellular CTLA-4 expression in patient's cells carrying the p.Gly109Glu variant was not affected (**Figure 5A**). Additionally, the percentage of CD80-binding ligand uptake was found to be reduced for cells carrying the variants p.Pro136Leu and p.Leu119Arg, which is consistent with the phenotype observed for other loss-of-function mutations; however, it was not reduced in the patient's Tregs carrying the p.Gly109Glu variant (**Figure 5B**). In these cells, the percentage of transendocytosis was comparable to that observed in healthy donors (**Figure 5B**). We conclude that the functional tests conducted by us were not suitable for showing the CTLA-4 impairment caused by the p.Gly109Glu missense mutation. Patient's PBMCs carrying the variant p.Val40Met were not available for functional testing.

Six of the 291 Investigated Patients Carry Mutations in *STAT3*

Monoallelic gain-of-function (GOF) mutations in *STAT3* were identified in six unrelated patients. Patients P154 (c.857A>C; p.Glu286Ala) and P170 (c.863A>C; p.Gln288Pro) carried mutations that were recently shown to have an increased DNA binding affinity and baseline activity in comparison to the wild-type STAT3 (41), whereas the mutation identified in P196 (c.839A>C; p.Gln280Pro) only caused a slightly increased basal transcriptional activity, which was strongly increased after stimulation. However, the extent and duration of phosphorylation, as well as the distribution of pSTAT3 within the cell, was comparable to the wild-type STAT3 levels (41). Patient P182 carried a known GOF mutation (c.2147C>T; p.Thr716Met), which has been previously identified in patients with enteropathy and autoimmune cytopenias (44, 45). Similarly, patients P182 and P196 presented with either hepatomegaly or splenomegaly and autoimmune cytopenia. P182 and P154 had low levels of IgA and IgG2 in serum and suffered from recurrent and severe respiratory tract infections. P182 presented with vitiligo, warts and mastoiditis; whereas P154 developed GLILD, diabetes, a hematological neoplasm and suffered from recurrent herpes. P170 had thrombocytopenia, but unfortunately additional clinical information was not available. The clinical manifestations exhibited in patients P154, P182 and P196 were compatible with the clinical phenotype observed in patients with Infantile-Onset Multisystem Autoimmune Disease 1 (IMAD1) [OMIM #615952] caused by GOF mutations in *STAT3*.

Moreover, we detected two previously unreported germline missense variants in *STAT3*. P101 harbors the c.1999G>T; p.Val667Leu mutation, which has only been reported as a somatic mutation associated with the development of a T-cell lymphoma in one patient (34). Patient P264, who also carried the known p.Ala181Glu variant in *TNFRSF13B*, was found to carry an undescribed variant (c.207C>A; p.Ser69Arg) in *STAT3*. Patient P101 had a history of decreased IgA levels in serum, autoimmune cytopenia, splenomegaly, enteropathy, ILD and recurrent pneumonias; whereas patient P264 suffered from recurrent respiratory infections, atopic dermatitis, mild lymphoproliferation, celiac disease, chronic diarrhea, and arthralgias. Furthermore, P264 presented with reduced levels of all immunoglobulin isotypes. T cells from P101 and P264 showed only a slight reduction in STAT3 phosphorylation compared to controls (data not shown); therefore, these two cases were classified as possibly solved.

Deleterious Biallelic *LRBA* Mutations in Two of the 291 Patients

We identified biallelic disease-causing mutations in two unrelated patients, who presented with very low B cell number, enteropathy, pulmonary disease and autoimmune features. LRBA is implicated in the regulation of CTLA-4 and cell survival as well as in endosomal trafficking (69–71). The four deleterious mutations identified include two novel nonsense variants: c.7370C>G; p.Ser2457* and c.5143C>T; p.Gln1715* in patient P059, and the known mutations: c.1420C>T; p.Gln474* (43) and c.2836_2839delTTTC; p.Glu946* in patient P173, who was already reported as Patient 1 (105–1) by Gámez et al. in 2016 (43). Further work-up by using flow cytometry showed severely reduced surface expression of LRBA in PBMCs from patient P059 in comparison to the healthy donor, thus suggesting that both alleles fail to produce any functional LRBA protein (**Figure 5C**).

Four Patients With *ADA* or *ADA2* (*CECR1*) Mutations Were Identified in our Cohort

Four patients were found to carry relevant mutations either in *ADA* or in *ADA2*. Patient P221 presented with severe B lymphopenia with slightly reduced T-cell counts and hypogammaglobulinemia, suffered from recurrent respiratory infections leading to pneumonias, bronchiectasis, asthma and ILD. Furthermore, she suffered severe bacterial and viral infections including meningococcal meningitis. She was found to carry compound heterozygous mutations in *ADA* comprising one published amorphic missense variant (c.911T>G; p.Leu304Arg) and one unreported variant (c.890C>T; p.Pro297Leu). Levels of ADA enzymatic activity were undetectable with a definite increase in intracellular metabolites, thus confirming the suspected pathogenicity of both variants (data not shown).

Moreover, we identified three unrelated patients carrying biallelic mutations in *ADA2*. Patient P048 - who suffered from recurrent infections, pancytopenia, livedo reticularis and hypoalbuminemia - was found to carry a novel homozygous deletion resulting in a frameshift mutation and premature stop codon (c.68_71delAAGA; p.Phe23Serfs*7). Further work-up confirmed low levels of ADA2 enzymatic activity (data not shown). Patient P198, who was previously described as Patient 2 by Schepp et al. in 2017 (37), carried the missense change p.Cys408Tyr and the splice-site variant c.542+1G>A in compound heterozygosity. Patient P141, previously described as Patient 4 by Schepp et al. (37), carried the homozygous missense mutation p.Gly47Ala (38, 39).

Hemizygous *BTK* Mutations in Three Male Patients

Three unrelated male patients were identified carrying hemizygous mutations in *BTK* (Bruton Tyrosine Kinase), encoding an essential kinase for development and maturation of B cells to antibody-secreting cells (72). The mutations in *BTK* included a previously described single-nucleotide deletion (c.757delC) (48) in patient P295 leading to a frameshift and premature termination (p.Val253Leufs*10) and two missense mutations: p.Arg62Cys and p.Gly575Cys, which were identified in patients P281 and P215, respectively. The variant p.Gly575Cys, to our knowledge, has not been previously reported. Further work-up to test the deleterious potential of the p.Gly575Cys mutation showed detectable BTK protein expression (**Supplementary Figure 4A**) but reduced Ca^{2+} flux in naive CD19+CD21+ B cells (**Supplementary Figure 4B**), despite normal phosphorylation of Igα, SLP65 and BTK itself (**Supplementary Figure 4C**). Ca^{2+} signaling is downstream of BTK phosphorylation suggesting that - despite normal BTK phosphorylation on Y551 - the signal transduction downstream of BTK seems to be affected in cells harboring this mutation.

Mutation Identified in *PIK3CD*

Patient P397 was found to carry the most commonly reported GOF mutation (c.3061G>A; p.Glu1021Lys) in *PIK3CD* (53–55). She presented with recurrent infections, lung disease, bronchiectasis and Hodgkin lymphoma, consistent with the clinical phenotype observed in patients with activated PI3K delta syndrome (APDS).

DISCUSSION

Early clinical and molecular diagnosis of patients with PAD could avoid suffering from repeated or chronic infections and subsequent organ damage. The heterogeneous underlying genetic etiology of PADs - and IEIs in general - and the interpretation of rare or novel variants with an atypical immune phenotype further challenge the establishment of a definitive diagnosis. Particularly, VUS are disappointing for both physicians and patients, when relying on genetic testing to confirm a suspected diagnosis.

In this study we summarize our findings using NGS and a targeted gene panel (TGP) approach to analyze the genetic background of a diverse cohort of 291 individuals who presented with selective or complete antibody deficiency. The use of NGS technology coupled with the results obtained from subsequent *in vitro* functional testing allowed us to evaluate 57 possibly relevant mutations and establish a possible or definite molecular diagnosis in 72 of the evaluated patients.

If we consider all possible and definite cases, the diagnostic yield for this cohort goes up to 24.74%, which is within the range of what other studies have reported (10 to 70%) on different cohorts of IEI patients employing various NGS approaches (**Supplementary Table 3**) (8, 11, 57, 73–77). The rates of positive hits between studies varies greatly based on the method used (WES or TGP), patient pre-selection and population, percentage of consanguinity, the number of selected genes, and filtering strategies. For example, in studies including pediatric patients with an early disease onset or patients born to consanguineous parents with a marked phenotype, the likelihood of identifying the underlying genetic defect - regardless of the sequencing approach - is higher than in studies including adult patients, patients from non-consanguineous families, or patients with a less clear phenotype.

Most of the genetic studies in PAD cohorts employing a TGP or NGS approach published thus far included a variable number of IEI-related genes, ranging from 17 to 623 (**Supplementary Table 3**). In our study, we analyzed up to 287 genes known to be essential for B cell development, differentiation and activation, as well as genes important for T cell function and genes involved in other critical signaling pathways of the immune system. We observed that most of the genetically diagnosed patients carry mutations in *TNFRSF13B, NFKB1 or CTLA4*, which collectively account for 72.2% in our cohort (**Figure 2C**). The identification of disease-relevant mutations in only 10 of the 287 studied genes may be biased, since not all genes were sequenced in an equal number of patients (**Figure 2A**); however, there were 20 genes that were screened in more than 250 patients. On the other hand, even though some genes, such as *ICOS or SEC61A1*, were screened at least in 280 patients, we did not find disease-relevant variants. It is however not surprising that screening of genes such as *LRBA* or *RAG2* (also screened in more than 280 individuals) only led to the diagnosis of two individuals, since these genes are found more frequently mutated in pediatric cohorts or in individuals born from consanguineous families (which are both under-represented in our cohort). Interestingly, we found one patient with relevant mutations in *ADA*, which are also more commonly identified in pediatric cohorts. *ADA* was only screened in half of the cohort (146 patients), which suggests that if this gene had been sequenced in the entire cohort, we might have detected additional mutations in our adult cohort. However, this could be true for other genes as well.

There are technical factors that certainly influence the diagnostic yield of NGS, such as the coverage of the target regions and the sequence reading depths. Low coverage increases the likelihood of missing possibly relevant mutations. The average coverage per run in this study was above 90% for almost all regions of interest, and reached 98% when using SureSelect designs (**Supplementary Figure 1B**). In our hands therefore, SureSelect performed better than HaloPlex regarding not only the total percentage of bases covered, but also the variability between samples of the same run, or between different runs (**Supplementary Figure 1B**). Low sequence reading depths can be a limiting factor in WGS or WES; however, in our study, this was not an issue as the mean reading depth per run was about 1000x, and the run with the lowest mean reading depth had 116x (**Supplementary Figure 1A**). We also observed that the use of SureSelect designs led to a more uniform distribution of sequencing reads between samples of the same run than the use of HaloPlex designs (**Supplementary Figure 1A**). Despite the limiting factor of pre-selected genes in TGP, the superior sequencing metrics that can be achieved using this technology compared to WES or WGS makes it a reliable, cost-effective and rapid first-line approach to diagnose patients with more typical phenotypes. On the other hand, the advantage of using WES or WGS instead of TGP can enable the genetic diagnosis of patients with pathogenic variants in less common or unexpected genes. The 219 individuals for whom we did not achieve a clear molecular diagnosis, despite having a good coverage for a broad number of PAD-associated genes, should therefore be subjected to WES/WGS to investigate whether they carry disease-causing variants in other immune-related genes not included in our TGP designs or in non-coding regions. Our preliminary findings using WES in an overlapping cohort show and increased 10-15% diagnostic yield compared to the use of TGP (unpublished data), which is a yield comparable to what others have observed for different singleton and trio cohorts (56, 78–80). A follow up WES study to examine many of the unsolved cases is currently in progress. Moreover, in patients with complex diseases (e.g. CVID) it is critical to consider that two or more subtle defects present in different genes might cause the phenotype. There is accumulating evidence that at least a subgroup of CVID patients likely have an oligogenic or polygenic origin rather than a monogenic cause (81). Our results confirm previous observations that an accurate genetic diagnosis cannot be made in about 70-80% of patients with PAD using only a TGP - reflecting the broad and complex clinical spectrum of these group of patients - and that further analysis, including WES, WGS, SNP-arrays or long-read sequencing are required to increase the diagnostic yield. Furthermore, gene-specific functional assays must be available, suitable and sensitive enough to confirm or reject the pathogenicity of a particular VUS.

Autosomal recessive (AR) disorders remain four times more common than autosomal dominant (AD) disorders among described IEI (6). Nonetheless, in this study most patients (63/72) were found to carry a disease-relevant mutation in a gene that follows an AD mode of inheritance (mutations in *TNFRSF13B*, *CTLA4*, *NFKB1, NFKB2, STAT3* and *PIK3CD* genes). Six patients were found to carry compound heterozygous mutations in genes following an AR pattern of inheritance and three males were found to have hemizygous mutations in genes associated with X-linked recessive disorders. This trend in the increase of AD defects has been observed before in cohorts of CVID patients from Western countries with non-consanguineous backgrounds in the last years (8, 11).

In line with previous reports in CVID, more than 29.2% of our positive or possible cases (**Figure 2C**) were found to carry known deleterious changes in *TNFRSF13B* (11, 57, 73), although mutations in this gene are currently considered risk alleles rather than disease-causing variants, as they are also found in asymptomatic carriers

(11, 82). Most of the candidate variants in *TNFRSF13B* found in this study (**Table 3**) have been repeatedly associated with some degree of antibody deficiency, compromised B cell function, higher risk of developing autoantibody-mediated autoimmunity and/or lymphoproliferation (9, 58, 83). In our cohort, variants in *TNFRSF13B* have been observed in 9.6% of patients. However, the exact same *TNFRSF13B* variants are present in approx. 2.8% of the healthy population. This points towards *TNFRSF13B* as a considerable risk gene for antibody deficiency and autoimmunity by the factor of 3.4x. Although the penetrance of this risk alleles in the general population seems to be rather small (0.0133%), the risk is approximately 35% within families with at least one PAD patient (46 of 133 *TNFRSF13B* mutation carriers from 34 multiplex families were affected by dysgammaglobulinemia; B. Grimbacher, unpublished data). This observation points either to an involvement of a second confounding genetic factor, or an environmental trigger at work in these affected families, but not in the many families with *TNFRSF13B* variant carriers without antibody deficiency. For the purpose of comparability to other publications in the field, we have decided to call the *TNFRSF13B* variants with a biological impact on TACI signaling (9) pathogenic.

Of note, we detected four patients (P002, P018, P219 and P264) each carrying known variants in *TNFRSF13B* and a second potentially pathogenic mutation in *CTLA4*, *STAT3* or *NFKB1*. We currently do not know whether the identified variants in *TNFRSF13B* (TACI) might influence or affect disease presentation and severity in patients with additional pathogenic mutations in genes associated with other well-defined IEI disorders. Due to the complexity of TACI-mediated signaling, more specific functional analysis in patients with multiple mutations is needed in order to determine the contribution of TACI variants to the overall phenotype. It is in fact conceivable that two (or more) "weakly hypomorphic" variants affecting the same signaling pathway at different steps might act synergistically to trigger a pathogenic mechanism, as some studies in CVID patients have begun to demonstrate (84).

Notably, 16 patients of the 72 possible/positive cases had candidate variants in two or more genes (**Table 2**). However, the significance of these variants in the pathogenesis of PADs is still undetermined and further experimental evidence is needed to clarify whether the presence of these additional variants may influence the course and severity of the disease. Until now, only a few genetic studies have reported on the VUS identified in their IEI patients. Yet we believe that additional variants of uncertain significance in critical genes should also be reported, particularly in patients with broad, complex or variable phenotypes, such as CVID, which might not be explainable by monogenetic defects.

Hypomorphic mutations in other IEI genes (*BTK, GATA2, IL2RG*, *JAK3*, *RAG1*, *RAG2*, etc.) have been previously found in patients with antibody deficiency and milder phenotypes or in CVID patients (85–90). Interestingly, P215, who presented with hypogammaglobulinemia, low circulating B cells, and impaired vaccine responses, carried a new hypomorphic variant in the kinase domain of BTK. Additional functional evaluations revealed mild defects in B cell activation but not in protein expression, suggesting residual function of BTK. Similar to our findings, patients with a late-onset of BTK insufficiency and less severe phenotype due to hypomorphic mutations in this gene have also been reported (88, 91). This case demonstrates the importance of also considering hypomorphic mutations in adult patients besides complete loss-of-function (amorphic) mutations.

In summary, this work highlights the need for careful evaluation of PAD patients, in order to provide a reliable molecular diagnosis and to initiate the most appropriate treatment. This evaluation should combine the clinical data and laboratory parameters with the genetic findings and functional proof from experimental assays in order to establish solid genotype-phenotype correlations and thereby reduce the number of VUS. We conclude that at least the following genes: *ADA*, *ADA2, BTK, CTLA4, LRBA, NFKB1, NFKB2, PIK3CD, STAT3* and *TNFRSF13B* should always be considered in any custom panel design intended to be used as a diagnostic test for patients with complete (reduction of all major immunoglobulin isotypes) or selective antibody deficiency.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because uploading the data is not part of the participants consent according to Art. 7 GDPR. Requests to access the datasets should be directed to michele.proietti@uniklinik-freiburg.de or bodo.grimbacher@uniklinik-freiburg.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee of the University of Freiburg, Germany. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JR-R and AC-O conceived, analyzed, interpreted the data and wrote the manuscript. JR-R, KH, HH, MF, and BK performed experiments and analyzed the results. AC-O and MP developed the internal database and bionformatics analysis pipeline. RK, KW, SE, and BG provided patient care, collected and provided clinical data, and commented on the manuscript. BG and MP designed and supervised the project, provided resources and edited the manuscript. All co-authors reviewed, commented and approved the final version of the 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.2021.786516/full#supplementary-material

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MINI REVIEW
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Good's Syndrome: Time to Move on From Reviewing the Past

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For seven decades, the pathophysiology of Good's syndrome (GS) has remained a mystery, with few attempts to solve it. Initially described as an association between hypogammaglobulinemia and thymoma, controversy exists whether this is a unique disease, or a subgroup of Common Variable Immune Deficiency (CVID). Recently, some distinguishing aspects of both syndromes have come to light reflecting fundamental differences in their underlying pathophysiology. GS and CVID differ in demographic features and immune phenotype. GS is found almost exclusively in adults and is characterized by a significantly reduced or absence of peripheral B cells. In CVID, which also occurs in children, most patients have normal or slightly reduced peripheral B cells, with a distinguishing feature of low memory B cells. Similarly, differences in T cell dysregulation and manifestations of hematologic cytopenias may further distinguish GS from CVID. Knowledge of the clinical phenotype of this rare adult immune deficiency stems from individual case reports, retrospective, and cross-sectional data on a few cohorts with a limited number of well characterized patients. The understanding of pathophysiology in GS is hampered by the incomplete and inconsistent reporting of clinical and laboratory data, with a limited knowledge of its natural history. In this mini review, we discuss current state of the art data and identify research gaps. In order to resolve controversies and fill in knowledge gaps, we propose a coordinated paradigm shift from incidence reporting to robust investigative studies, addressing mechanisms of disease. We hope this novel approach sets a clear direction to solve the current controversies.

Keywords: Good's syndrome, CVID, immune deficiency, hypogammaglobulinemia, thymoma

INTRODUCTION

Good's syndrome (GS) was initially defined as a rare association of thymoma, invasive bacterial infections and hypogamma globulinemia, diagnosed in 40 to 60-year-old adults (1). It is one of the most unique, yet under-investigated immunodeficiencies. The initial definition of GS created a mindset that it is a subset of Common Variable Immunodeficiency (CVID) with thymoma. Subsequent evidence of T cell deficiency, autoimmune features, and myelodysplastic manifestations, suggest a more complex clinical picture. As a result, modifications to the original diagnostic criteria have been proposed to exclude non-immunodeficient overlap syndromes (2). This proposed paradigm shift stems from the lack of an established underlying etiology. Since first described by Dr. Robert Good in 1954, there has been a paucity of literature on the subject with a lack of clinical awareness, resulting in an inadequate characterization of this disease. A turning point in our understanding came following the discovery of B cells in 1960. It then became apparent that this syndrome included reduced or absent mature B cells (3). In the latest review of GS, four cohort studies reported a total of 44% of individuals who had complete absence of peripheral B cells, while 50% had low levels (4). Thus, it is appropriate that reduced or absent B cells be accepted as an additional diagnostic criterion for GS (5, 6). Furthermore, the focus on B cell lymphopenia may lead to understanding the underlying etiology.

The discovery of other thymoma-related disorders of immunity led to a blurring of the classic definition of GS. Thymoma associated disorders, including autoimmune manifestations, giant cell myocarditis, myasthenia gravis, absolute lymphocytosis, and also isolated T cell immunodeficiency of unknown pathogenesis, have created a diagnostic dilema by inappropriately defining some of these patients as having atypical GS (6–9). In this review, we summarize existing knowledge, identify the controversies and "unknowns" with possible avenues of investigations on its immunopathology. We specifically focus on the importance of clearly defining GS as an immune deficiency and stress the importance of B cell lymphopenia.

CONTROVERSIES REGARDING UNDERLYING IMMUNE PATHOLOGY:

Is GS a Subset of CVID?

Defects in B cell differentiation and antibody production comprise the largest group of inherited disorders of immunity (10). From a B cell perspective, GS fits between two sub-categories of primary antibody deficiencies (11, 12). The first is characterized by absent or significantly reduced numbers of B cells and all serum immunoglobulins, such as X-linked agammaglobulinemia, λ5 deficiency, BLNK deficiency, PIK3R1 deficiency, and Igα/β deficiency. The second subcategory, the CVID phenotype, is characterized by a history of recurrent bacterial sino-pulmonary infections, low or normal numbers of peripheral B cells with a moderate to severe reduction in at least 2 immunoglobulin isotypes and poor responses to vaccines. In contrast to patients with CVID, only <5% of patients with GS have normal levels of B cells (4, 13). GS is traditionally grouped with antibody deficiencies, where 100% GS patients have low serum IgG levels and 86% have low IgA and 92.6% low IgM (4). While CVID patients may have defects in peripheral B cell survival, differentiation, and antibody production, GS patients are different from CVID, and are similar to those with agammaglobulinemia in that they lack B cells, suggesting a defect or interference in lymphopoiesis during early B cell development (See **Table 1**).

TABLE 1 | Key features of GS with contrasts to XLA and CVID.

	CVID	XLA	GS
Prevalence	1 per 25,000-1 per 50,000 (14)	1 per 1,400,000 (15)	1 per 700,000 (16)
Diagnostic Criteria			
Thymoma	–	–	+
Hypogammaglobulinemia	+	+	+
Presence of peripheral B cells	Normal to moderate for 90% of patients (13)	Absent for 100% of patients (17)	Significantly reduced or absent for 99% of patients (16)
Age			
Proportion diagnosed in childhood	20% (18)	100% (15)	<1% (19)
Age range at presentation	20-40yrs (18)	0-2yrs (20)	40-60yrs (21)
Infections			
Invasive bacterial	+++	+++	+++
Opportunistic	+	–	++
Associated clinical conditions	ITP, AIHA, lymphadenopathy, splenomegaly (22)		PRCA, myasthenia gravis, lichen planus (16)
Frequency of autoimmune complications	~20–30% (22)		>50% of cases (4)
Genetic cause identified	Identified in 10% of cases (TACI, BAFF-R, CD40, CTLA) (14)	BTK (23)	Unknown

(-/+): absence/ presence of characterstic; (++/+++): increasing frequency.

A recent study of bone marrow samples from GS patients, found that the block in B cell differentiation was at a different stage than in agammaglobulinemia patients with defined monogenic causes (XLA, μ-chain-, CD79 or BLNK deficiency) (24). While most B cell progenitors (BCPs) in the agammaglobulinemia patients were arrested at the pre-B-1 and pre-B-II stage, due to defective pre-BCR signaling, BCPs of GS arrested at the earlier Pro-B cell stage. GS patients also had a severe reduction in total BCPs compared to healthy controls and agammaglobulinemia patients. It is unlikely that a germline defect in B cell differentiation is the underlying cause because of the late onset, and reports of monozygotic twins discordant for GS (25, 26).

In addition to recurrent bacterial infections, GS patients experience an increased frequency of opportunistic infections (OIs) (mucocutaneous candidiasis, Pneumocystis jirovecii) and reactivation of latent viruses (cytomegalovirus, varicella zoster, human polyoma virus 2, herpes simplex virus) (27–30). The presence of OIs is diagnostic of significant T cell deficiency and thus, defines the syndrome as a combined immune deficiency. OIs are not a hallmark of antibody deficiencies such as XLA and CVID, however, a small subset of patients with CVID do report such infections (31). It is yet unclear if defects in cellular immunity are a feature of all GS patients or similar to CVID, they only occur in a subgroup. T cell dysregulation has been extensively studied in subsets of CVID with a wide and heterogenous set of abnormalities. Despite a higher proportion of GS patients with cellular immunodeficiency, in-depth investigations on T cell function have not been carried out to the same extent as in CVID (16).

Is It an Inborn Error of Immunity or a Secondary Antibody Deficiency?

Monogenic defects occur in some patients with CVID, involving pathways of B cell development and differentiation, and maintenance of germinal centers (11, 32). No disease-causing variants have been associated with GS, with only three independent genetic studies being conducted so far. Out of eight patients reported, two were found to have a mutation in TACI and one individual has been reported to carry two missense mutations in BAFF-R (33–35). BAFF-R and TACI, members of the Tumor Necrosis Factor Receptor (TNFR) superfamily, are involved with B cell maturation and homeostasis, and variants of these genes have been associated with CVID. Although animal models indicate loss of function mutations in TACI which result in peripheral B cell deficiency, human single nucleotide variants are not associated with peripheral B lymphocyte changes (36). Presence of mutated variants do not confirm that these genes are in fact disease causing or even susceptibility increasing.

Furthermore, there are arguments to suggest that unlike XLA or other agammaglobulinemia disorders, GS may not be driven by a mono or polygenic cause. Most cases of CVID and GS are adult-onset. The age of CVID diagnosis has a bimodal presentation: ~20% being diagnosed during early childhood and the other set, usually between ages 20-40, often with a prodrome of recurrent infections years before their diagnosis (18). GS patients, almost exclusively, report a healthy childhood and adolescence, with the diagnosis of both thymoma and hypogammaglobulinemia, occurring between 40-60 years of age. In contrast to these adult antibody deficiencies, most cases of congenital agammaglobulinemia are diagnosed in early infancy, after 6 to 9 months, when most of the maternal antibodies have been lost (See **Table 1**) (17). The very late onset of GS, in patients who also have absent peripheral B cells and arrested progenitor B cells, highlights the importance of age as a risk factor. However, studies have not determined the roles of incomplete penetrance or mosaicism. Understanding the mechanism of disease in GS may also shed light on the role of age-dependent epigenetics in B cell lymphopoiesis.

GS is postulated to be a secondary immune deficiency (i.e. thymic tumor induced hypogammaglobulinemia) (37). The diagnosis of thymoma is usually an incidental finding or part of the diagnostic investigations for suspected myasthenia gravis, or recurrent pneumonia and bronchitis (38). In 90% of GS cases, the thymomas reported are benign and localized. One systematic review reported the spindle cell as the most common variant (WHO classification A), while others reported > 50% of cases are mixed thymomas (WHO classification AB) (21, 29). In either case, no association was found between thymoma type and type of opportunistic infections or secondary autoimmune complications. The diagnosis of thymoma can precede, occur concurrently, or follow the diagnosis of the hypogammaglobulinemia, at relatively equal frequencies (21, 29, 39).

In one large retrospective survey, the median age of GS diagnosis was 58 (51-62 years), and a median interval of four years between diagnosis of thymoma and that of hypogammaglobulinemia, irrespective of the sequence of presentation (29). Thymectomy does not reverse the immune or hematologic abnormalities in these patients (27). The timing and relative non-impact on the hypogammaglobulinemia can lead to the impression that the thymoma itself does not drive the B cell depletion in GS but is simply another clinical manifestation of the unknown immunopathology.

Autoimmune Complications vs. Bone Marrow Dysplasia

Although both GS and CVID have hematologic manifestations, the underlying mechanisms differ. In GS, Anemia is seen in 50 to 86% of patients. Pure Red Cell Aplasia (PRCA) is the most common cause, along with aplastic, hemolytic, and pernicious anemia and myelodysplastic syndromes (4). These manifestations are indicative of bone marrow failure. In contrast, the most common autoimmune conditions in CVID, are immune thrombocytopenic purpura (ITP) and hemolytic anemia (AIHA), both are antibody mediated (40). Bone marrow dysfunction may be responsible for other hematologic defects associated with GS including lymphocytopenia, CD4 lymphopenia, neutropenia and eosinopenia (21). Lymphadenopathy and splenomegaly, seen frequently in patients with CVID, are rare amongst GS patients. The contrasting features of these two predominantly antibody deficiencies are shown in **Table 1** and may provide more insight on the intersection of genetics, autoimmunity, and inborn errors of immunity (41, 42).

Given that no genetic defect in B cell differentiation has been demonstrated in GS, there may be intrinsic and/or extrinsic factors driving the B lymphopenia. An oligoclonal expansion of a subset of CD8 T cells with a vβ8 T cell receptor (TCR) in the bone marrow of patients with a thymoma and B lymphopenia, has been reported. However, this expanded subset was not seen in the same patients' peripheral blood lymphocyte population (43). Direct sequencing revealed a conserved CDR3 motif in the vβ8 TCR (SF/LGXGXNXXQ/LH/Y) suggesting that this could be an antigen-specific response to either an unknown pathogen or an autoimmune targeting of B cell progenitors. Several studies have shown that the thymic tumor microenvironment can cause aberrant maturation of T cell precursors and alter the T cell subset composition in the blood, but most studies are limited to myasthenia gravis. Additional hypotheses suggest the role of autoantibodies and limitin, an interferon-like cytokine produced by bone marrow stromal cells, in suppressing or skewing the differentiation and growth of B cell precursors (39).

A few small studies have reported that GS peripheral blood lymphocytes can suppress both pre-B cell differentiation and peripheral B cell differentiation to plasma cells, and subsequent immunoglobulin synthesis. Since GS patients have almost no peripheral B cells nor pre-B cells at diagnosis, their T cells or lymphocytes were co-cultured with either bone marrow cells or peripheral B cells of allogenic healthy controls. The lack of HLA matching between donors could imply that the suppressive effect was due to an allogenic response to non self MHC (25, 44–46). Of interest, in one study, the suppressive nature of lymphocytes on B cell differentiation and function, was still observed when the patient's cells were co-cultured with those of their monozygotic HLA identical twin (25). More work needs to be done to ascertain if CD8 T cells are in-fact the drivers of the B cell lymphopenia of GS and if it is mediated by contact-based cytotoxicity or functional suppression through secretion of a soluble factor. The development of an immune response targeting self could be multi-factorial. In addition to thymic skewing of T cell subsets, an environmental "hit" could also cause an expansion of suppressive clonal lymphocytes. Several viruses and bacteria have been postulated to induce auto-immunity through molecular mimicry, epitope spreading, bystander activation and cross-reactive antibody production (47, 48). Identification of a common pathogenic agent in patients would be radical, as this would reclassify GS as an induced autoimmune disorder leading to a secondary immune deficiency and can create a shift in treatment paradigm.

DISCUSSION: RESEARCH GAPS & MOVING FORWARD

In a 2003 detailed review on GS, extensive recommendations were made on investigations required to fill the gaps in knowledge (39). Almost two decades later, these gaps remain strikingly evident (see **Table 2**). The existing literature on GS, consists mainly of individual or small series of case studies. These have been focused on hospitalized patients with admissions for severe infection, short-term clinical treatment, and outcomes. To date, only two cohort studies have been conducted with only one being prospective in nature (16, 29). This, of course, reflects the very low prevalence of 1.5 cases per 1,000,000 individuals in a population per year (16). It is encouraging that patients are being recruited through primary immunodeficiency registries. However, longitudinal studies, as those conducted for CVID, are required to delineate the natural history of the GS. Moving forward with limited patient numbers, concerted multicenter efforts should include consensus laboratory and clinical investigations. A dedicated database and biobank should improve power and quality of genetic analysis and *in-vitro* assays.

The current evidence on genetics can neither confirm nor rule out that there is a monogenic cause underlying GS. It is useful to

TABLE 2 | Existing knowledge gaps regarding Good's syndrome and proposed solutions.

Knowledge Gaps regarding GS	Proposed solutions
Incomplete definition of GS	- Reaching consensus on the diagnostic criteria
	- Incorporation of B cell lymphopenia in the criteria
	- Validation and use of other biomarkers (e.g.CD247) to rule out other differential diagnosis with other thymoma associated phenotypes (6)
Lack of published natural history of disease	- Multi-centre longitudinal studies
	- Case reports detailing clinical events at multiple time points
Low power genetic screens and *in-vivo* assays	- Increase awareness and patient recruitment at a multinational level
	- Open access of real time results
	- Establishment of a dedicated GS database
	- Establishment of a blood and tissue biobank
Lack of uniform and detailed immune characterization	- Consensus on immunophenotyping
	- Longitudinal bio-marker sampling, in good health and when symptomatic
	- Report laboratory ranges for healthy and other immune deficiency controls
	- Promote the use of functional immune assessments (cytokines, lymphocyte proliferation)
Unvalidated hypotheses regarding etiology or mechanism of disease	- Additional bioinformatics investigations - whole exome sequencing, RNA seq, HLA typing
	- Screens for autoantibodies and soluble factors that interfere with B cell lymphopoiesis (e.g., limitin)
	- Additional laboratory investigations-culturing of thymus, bone marrow or peripheral PBMCs

screen diagnosed GS patients for all known immune deficiency associated genes and make the results available in open access, even if no variant of significance is found. This will help narrow down potential candidate alleles that possibly occur in GS patients. Given the age of onset, the defect may be at a translational or epigenetic level. It is therefore important that whole exome sequencing, protein expression quantification and CGH microarray (looking for micro-duplications and micro-deletions) be considered. A particular tissue of interest for study is the thymus at removal. A multiomic approach should be taken, using targeted panels or whole exome sequencing, looking for somatic mutations, as well as RNA sequencing, of thymoma cells. GS thymoma cells should be compared to the appropriate controls; healthy thymic tissue or other thymoma associated diseases such as myasthenia gravis. This may provide insight on the variability in histological type of the thymomas reported in case studies (Type A vs Type AB). Similar approaches can also be taken with bone marrow samples. The purported role of CD8 T cells provides rationale for HLA typing, which has been seen to be skewed in other diseases such as CVID and myasthenia gravis (36, 49).

Adequate GS immune characterization is lacking, and if present, is difficult to reproduce. Less than half of case reports provided immunophenotyping and if available was not in a uniform fashion (21). Some reported absolute cell numbers, others reported percentages (39). Additionally, many evaluations were on a single occasion in the disease course or at an age not stated. Nor can we assume consistency of data for all individuals reported. Most case studies were of hospitalized individuals with significant infections, possibly skewing the laboratory findings. Often results were provided without context (such as normal ranges for healthy age matched controls) or comparison with appropriate disease controls (such as individuals with CVID). Only a single study compared individuals with CVID and GS prospectively, but chose to focus on clinical infections, and only included a single immune investigation at an unspecified time-point (16). There is a need for an increased awareness of this syndrome. Once diagnosed, patients should be followed regularly with standardized clinical evaluations. Given the importance of myelodysplasia, trends in neutrophils, platelets, and red blood cells over time should be monitored on follow-up and captured in a common database. Early detection documenting the onset of lymphopenia, absence of B cells and neutropenia would improve our understanding of the progressive nature, immune deficiency, and dysregulation in this syndrome.

There is a need for robust in-depth extended immune phenotyping, including vaccine challenge for humoral and cellular responses. *In-vivo* assessment using anergy screens, have been rarely reported. Beyond *in-vitro* mitogenic lymphocyte stimulation with lectins such as PHA, it is important to assess responses to microbial recall antigens, as well as post vaccination to neoantigens (SARS Cov-2). As *in-vitro* stimulation alone may not predict the susceptibility of select patients to opportunistic infections, measuring thymic output and diversity of the CD4 and CD8 TCR repertoire may also offer predictive value given the para-thymic nature of this syndrome. The multiple hypotheses arguing the autoimmune mediated repression of the proliferation of pro-B lymphocytes must also be tested. A first step in this direction would be screening for autoantibodies or other suppressive factors such as limitin in patients with thymoma (see **Table 2**).

Although informative, case studies of a cross sectional nature and subsequent reviews of the literature, have shed little light on the etiology and the underlying immunopathology. Since the initial reporting of GS, improvements in laboratory technology and bioinformatics, have been crucial in solving many medical mysteries. With the increase and almost instantaneous global sharing of scientific knowledge, we recommend sharing of databases and biobanks, given that these are quintessential assets when dealing with limited patient numbers. We foresee that if these tools and cooperative agreements are optimally deployed, knowledge gaps in GS can be closed, accelerating the understanding of the pathophysiology of this rare disease.

AUTHOR CONTRIBUTIONS

CT conceived and supervised the mini review. AK reviewed the literature and drafted all sections of the article. CT and RA revised and edited the draft. All authors read and approved the final article.

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OPINION
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Common Variable Immunodeficiency Disorders as a Model for Assessing COVID-19 Vaccine Responses in Immunocompromised Patients

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INTRODUCTION

COVID-19 has had a disastrous impact on the world with over 5 million deaths, hundreds of millions infected and many more plunged into poverty. COVID-19 has affected almost all countries. The origin of the virus is the subject of ongoing study (1–3).

SARS-CoV-2 initially infects the nasal mucosa. The Spike (S) glycoprotein engages cell-surface ACE2. Host proteases including TMPRSS-2 cleave the S glycoprotein, allowing the S2 subunit to fuse with the cell membrane (4). The viral genome is then able to hijack cellular organelles leading to production of daughter virus particles.

In the initial asymptomatic nasal phase, the innate immune system is silenced resulting in an exponential increase in viral progeny. SARS-CoV-2 deploys several mechanisms to evade cytoplasmic viral sensors. Following the nasal phase, the virus infects the lungs, probably by microaspiration from the nasopharynx and stomach (5, 6). Patients suffering pneumonitis experience increasing dyspnoea and have elevated inflammatory markers.

The smaller percentage entering the systemic phase suffer acute respiratory distress syndrome (ARDS) and multiple organ dysfunction. Increased d-dimers indicate a risk of arterial and venous thromboembolic disease. In spite of invasive ventilation and extracorporeal membrane oxygenation, mortality remains very high in such patients.

PATIENTS AT INCREASED RISK

There is a high case fatality rate in the elderly (7). In addition, patients with comorbidities including obesity, diabetes, hypertension, coronary artery disease, malignancy, renal and pulmonary disease are at increased risk of adverse outcomes (7–9). Patients of Black, Hispanic and South Asian origin also have a higher case fatality rate. Inequitable access to healthcare is at least partly responsible for these ethnic differences (10, 11).

Current data also suggests individuals with some immunodeficiency disorders are at increased risk of severe outcomes (12, 13). Patients with innate immune defects and T cell disorders are at greater risk than healthy individuals (14–16). In contrast, most studies indicate patients with X-linked agammaglobulinemia (XLA) without comorbidities appear to be at lower risk, inferring antibodies can in some circumstances be detrimental (17–20). Some authors are however less certain about the protective effect of XLA in COVID-19 outcomes (21, 22).

Immunocompromised patients are at risk of Chronic COVID-19, a dangerous stalemate between SARS-CoV-2 and impaired cellular immunity (23). Patients with Chronic COVID-19 can shed virus for months before either succumbing to or recovering from the infection. Such patients are vulnerable to intra-host viral evolution which could result in variants of high consequence (24). This is a public health emergency and prevention of Chronic COVID-19 is of the utmost priority.

RESPONSE TO COVID-19 VACCINES

Vaccines have proved effective in mitigating COVID-19. Vaccines do not prevent breakthrough infections, but markedly reduce the risk of a destructive immune response (**Figure 1**) (25). Hospitalisations and deaths from COVID-19 have dramatically decreased following vaccination. Most vaccinated patients dying from breakthrough infections are elderly or those with comorbidities. Vaccinated patients have variable levels of antibodies to the S glycoprotein at the time of breakthrough infections (26). There is no specific antibody level, which reliably prevents breakthrough infection (27, 28). The S glycoprotein is post-translationally modified with carbohydrates and antibody responses are less durable. In many studies antibody levels decrease six months after vaccination (29).

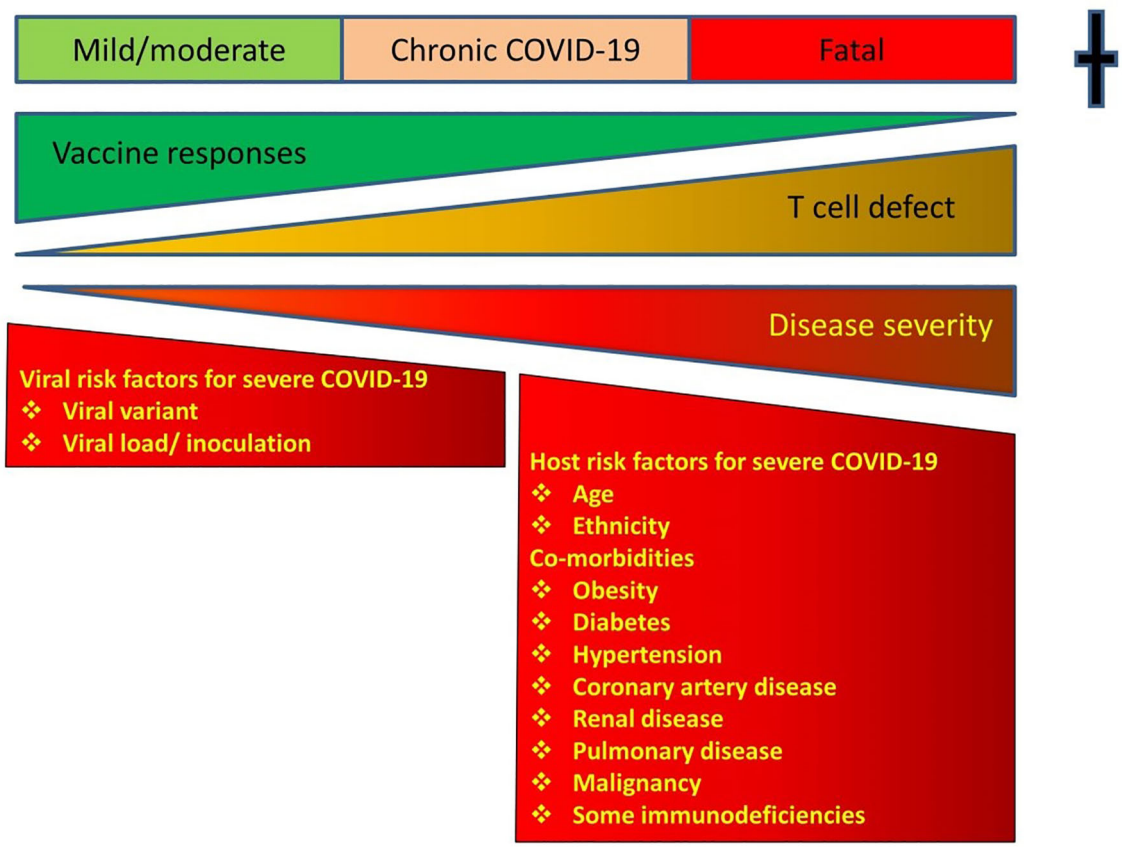

FIGURE 1 | Showing the relationship between T cell defects, responses to vaccines and outcomes. Vaccines shift the disease severity to the milder end of the spectrum. Instead of fatal disease, vaccinated patients with T cell defects will have a milder version of the infection. There is a risk of Chronic COVID-19 in immunodeficient patients and a vigorous T cell response to vaccines shifts the disease severity to the milder end of the spectrum. COVID-19 disease severity according to WHO criteria.

In contrast, memory T cell responses to vaccines correlate with protection (25, 30). Diagnostic T cell assays can be measured on different platforms, depending on the expertise of the laboratory (31). Current COVID-19 vaccines are regarded as T cell dependent and cellular responses are more durable, indicating that waning antibody levels underestimate the duration of protection (32). The S glycoprotein has strong adjuvant properties for cellular immunity, increasing its immunogenicity (and reactogenicity). Current COVID-19 vaccines do not require an additional adjuvant.

COMMON VARIABLE IMMUNODEFICIENCY DISORDERS AS A MODEL OF IMMUNOCOMPROMISED INDIVIDUALS

Common Variable Immunodeficiency Disorders (CVID) are the most frequent symptomatic primary immune defect in adults and children (33, 34). By definition, the cause of CVID is not known (35–37). In some patients an underlying genetic defect is causative (38). If a causative defect is identified, these patients are considered to have a CVID-like disorder and are removed from the broad umbrella diagnosis of CVID. In non-consanguineous populations approximately 25% have an underlying genetic defect, mostly autosomal dominant disorders (39, 40). In consanguineous societies a much higher number have autosomal recessive disorders (41).

Currently there are three sets of diagnostic criteria for CVID in common use (35–37). The original European Society of Immunodeficiencies/Pan-American Group for Immunodeficiency (ESID/PAGID) 1999 Criteria required significant hypogammaglobulinemia (IgG 2 sd. below the mean) with either impaired vaccine responses or absent isohemagglutinins (42). These were deemed difficult to use (43). In 2013 new diagnostic criteria were proposed with a lower IgG threshold (5 g/L) and vaccine responses beyond protection, to those of normal persons (35). These criteria also contained many of the more recent discoveries including reduction in switched memory B cells and genes predisposing to CVID. In contrast to the previous criteria, impaired vaccine responses were not mandatory for the diagnosis. The revised ESID registry criteria were published in 2014 (36). These were very similar to the Ameratunga et al., criteria (35), but maintained the higher IgG threshold (2 sd. below the mean) and protective vaccine responses of the original ESID/PAGID 1999 Criteria. In 2016 the International Consensus (ICON) document was published (37). Like the original ESID/PAGID 1999 criteria, poor responses to vaccine were mandatory in the ICON 2016 Criteria.

CVID and CVID-like disorders have a spectrum of B and T cell defects. The ESID 2014 and ICON 2016 criteria exclude patients with severe T cell defects, who were deemed to have late onset combined immunodeficiency (LOCID) based on reduced naïve $CD4^+$ T cell proportions (<10% $CD4^+$ T cells) (36, 37). It has however been suggested patients with LOCID should remain within the broad spectrum of CVID and CVID-like disorders (44). Individuals within the same family, carrying the identical *NFKB1* mutation, had widely differing immune defects. One brother was in excellent health, while his sister suffered multiple autoimmune complications and malignancy. She met the criteria for LOCID because of reduced T cell subsets and died prematurely from hepatic failure (45, 46).

CVID AS A MODEL OF VACCINE CHALLENGE RESPONSES IN IMMUNOCOMPROMISED PERSONS

Although not mandatory in the Ameratunga et al., 2013 or ESID 2014 Criteria, vaccine challenge responses are an integral part of the diagnostic work-up of patients with suspected CVID. CVID and other antibody deficiency disorders can serve as a useful model for both susceptibility to COVID-19 as well as responses to vaccines. In contrast to CVID, vaccine challenge responses are not routinely undertaken in patients suffering from secondary immunodeficiency disorders for either diagnosis or prerequisites for therapy. These patients receive subcutaneous or intravenous immunoglobulin (SCIG/IVIG) replacement based on either profound hypogammaglobulinemia or if they have modest hypogammaglobulinemia with breakthrough infections.

Two recent studies have explored the responses to vaccines in patients with hypogammaglobulinemia as well as CVID. In the New Zealand hypogammaglobulinemia study (NZHS), asymptomatic patients with hypogammaglobulinema (aHGUS) were noted to have an excellent prognosis (47). In this long-term prospective study, only one patient experienced progressive hypogammaglobulinemia requiring SCIG/IVIG. The majority have remained well for over a decade. In contrast, those with symptoms (sHGUS) had a mixed prognosis. Many experienced progressive deterioration culminating in SCIG/IVIG treatment. Vaccine challenge responses in the two groups were indistinguishable. Importantly, both groups had excellent responses to HIB and tetanus toxoid, both T cell dependent antigens. In contrast, responses to diphtheria toxoid and Pneumovax were muted. Poor responses to diphtheria toxoid are common, particularly in the elderly. Pneumovax responses are T cell independent.

A similar outcome was noted in the New Zealand CVID Study (NZCS) (48, 49). Most patients meeting criteria for CVID had excellent responses to tetanus toxoid and HIB. As in the NZHS, the responses to diphtheria toxoid and Pneumovax were suboptimal. This indicated T cell responses were preserved for at least some antigens in CVID. Recent studies confirm many CVID patients may generate protective responses to COVID-19 vaccines (50, 51).

APPROACH TO IMMUNOCOMPROMISED PATIENTS

The most important outcome of COVID-19 vaccination is a balanced, co-ordinated cellular immune response to the virus (25, 30). This implies at least some T cell function is required for vaccine efficacy (50). Given what was noted in the NZHS and

NZCS, COVID-19 vaccines will provide at least partial protection in most immunocompromised patients. This is a strong argument for vaccinating these patients and monitoring their T cell responses to SARS-CoV-2 (50–52).

Immunocompromised patients should be individually assessed to determine the degree of cellular immune deficiency. The extent of cellular impairment can be ascertained by the types of infections as well as laboratory tests including T cell subsets and their *in vitro* function. The nature of the underlying disorder and therapy may also help identify impaired cellular immunity. Such an individualised approach can sometimes lead to unexpected findings. Patients treated with rituximab are more susceptible to COVID-19 than those with XLA (53–55). This may be because of the underlying disorder or because of the use of additional immunosuppressive agents.

The WHO, UK and other countries are now advocating a three dose primary COVID-19 immunisation program for immunocompromised persons. This may improve memory T cell responses to the vaccine (56, 57). It remains to be determined if heterologous primary immunisation, with mRNA and adenovirus-based vaccines generates a robust cellular response, as seen with humoral responses in healthy individuals (58). Again, monitoring T cell responses following vaccination will provide reassurance (50, 55).

It will be difficult to monitor antibody responses to COVID-19 vaccines if patients are on SCIG/IVIG. Most plasma donors have high titres of SARS-CoV-2 antibodies from either infection or vaccination (59). SARS-CoV-2 memory B cells could be quantified as a measure of humoral immunity in patients on IVIG/SCIG. These responses have been quantitated in PID patients receiving the influenza vaccine (60).

Antibody responses to the S glycoprotein are T cell dependent. In patients who are not on SCIG/IVIG, a good antibody response could be interpreted as a satisfactory cellular response to the vaccine. In those who have poor antibody responses, it is still possible they have protective T cell responses (50, 55). Many healthy persons failed to seroconvert but had robust T cell responses to SARS-CoV-2 (61). There have been calls for development of diagnostic T cell assays for SARS-CoV-2, which would be very useful for diagnosis or evaluating vaccine responses in immunocompromised patients (31, 61).

The best current advice is for immunocompromised patients including those with antibody deficiency to have at least three primary vaccinations and have their T cell responses measured (56, 57). If there is failure to generate cellular immunity to SARS-CoV-2, these patients should be advised to shelter in place until more effective therapeutics and vaccines are developed for COVID-19. The recent development of antiviral drugs by Merck (molnupiravir) and Pfizer (paxlovid) is encouraging. Until these drugs are widely available, patients with sub-optimal memory T cell responses remain at risk of severe outcomes or Chronic COVID-19 (**Figure 1**). If there is waning cellular immunity they should receive boosters. In the absence of a diagnostic T cell assay for SARS-CoV-2, booster COVID-19 vaccines could be routinely considered every 6 months or sooner.

AUTHOR CONTRIBUTIONS

RA wrote the first draft. All other authors contributed to editing the manuscript.

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A SARS-CoV-2 T cell assay has not been implemented in NZ.

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The Immune Response to SARS-CoV-2 Vaccination: Insights Learned From Adult Patients With Common Variable Immune Deficiency

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CVID patients have an increased susceptibility to vaccine-preventable infections. The question on the potential benefits of immunization of CVID patients against SARS-CoV-2 offered the possibility to analyze the defective mechanisms of immune responses to a novel antigen. In CVID, as in immunocompetent subjects, the role of B and T cells is different between infected and vaccinated individuals. Upon vaccination, variable anti-Spike IgG responses have been found in different CVID cohorts. Immunization with two doses of mRNA vaccine did not generate Spike-specific classical memory B cells (MBCs) but atypical memory B cells (ATM) with low binding capacity to Spike protein. Spike-specific T-cells responses were also induced in CVID patients with a variable frequency, differently from specific T cells produced after multiple exposures to viral antigens following influenza virus immunization and infection. The immune response elicited by SARS-CoV-2 infection was enhanced by subsequent immunization underlying the need to immunize convalescent COVID-19 CVID patients after recovery. In particular, immunization after SARS-Cov-2 infection generated Spike-specific classical memory B cells (MBCs) with low binding capacity to Spike protein and Spike-specific antibodies in a high percentage of CVID patients. The search for a strategy to elicit an adequate immune response post-vaccination in CVID patients is necessary. Since reinfection with SARS-CoV-2 has been documented, at present SARS-CoV-2 positive CVID patients might benefit from new preventing strategy based on administration of anti-SARS-CoV-2 monoclonal antibodies.

Keywords: immunization, common variable immune deficiency, vaccine, antibodies, SARS-CoV-2

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INTRODUCTION

The Committee of Experts on Primary Immunodeficiency of the International Union of Immunological Societies (IUIS) has included vaccination both as a diagnostic tool to assess the specific antibody response to protein and/or polysaccharide antigens and as a means of prevention (1).

The type and severity of the immunodeficiency determines the efficacy of vaccines, with varying levels of impairment, ranging from normal as in immunocompetent individuals, to incomplete or even absent. The degree of immunodeficiency and the specific defect in antibody production are variable in common variable immunodeficiency (CVID) (2) and each patient should be studied as unique in terms of cellular and humoral responses.

The decision to vaccinate a patient must include a risk and benefit assessment to ensure maximum protection and avoid adverse events. In addition, other factors, including the type of vaccine, the interval between administrations, and the time between gamma globulin administration and vaccination, must also be taken into account in defining an immunization strategy.

Here, we provide an updated perspective on the pathogenesis of CVID based on the studies performed on immune responses to vaccines with the aim to evaluate whether the immunization strategy for adult patients with CVID is effective. Studies on the potential benefits of immunization against SARS-CoV-2 offered the possibility to investigate the impaired pathogenic mechanisms of response to a novel antigen in patients with CVID.

SUSCEPTIBILITY TO VACCINE-PREVENTABLE INFECTIONS

CVID patients have an increased susceptibility to vaccine-preventable infections. Although the predominant infections are of bacterial origin, viral infections caused by rhinoviruses, parainfluenza, noroviruses, and herpesviruses, including varicella herpes zoster (VZV), adenovirus, respiratory syncytial virus, that, in turn, play a role in driving an underlying inflammatory condition, are reported in CVID (3). SARS-CoV-2 infection also has been reported in CVID, since the beginning of the pandemics, with a low prevalence possibly due to the choice of most physicians to inform CVID patients early about safety measures, and to switch most patients to home therapy and remote assistance (4). Within SARS-CoV-2 infected patients with inborn errors of immunity, CVID patients represent the largest proportion since CVID is the most commonly diagnosed/reported IEI (5), have more comorbidities and older age (5). Moreover, CVID patients have an increased risk for prolonged infections and a low probability to clear viruses as it has been demonstrated for SARS-CoV-2 (6) as well as for the poliovirus, in particular when the number of peripheral blood B lymphocytes is low (7). Genetic differences contribute to individual variations in the immune response to pathogens and in the response to immunization. Pathogenic loss-of-function or gain-of-function heterozygous variants have been reported to be associated with CVID. However, their functional relevance for susceptibility to infection and response to vaccination remains to be clarified (8). Unfortunately, genetic causes of most CVID cases remain undefined, and the diagnosis is predominantly based on hypogammaglobulinemia with impairment of antibody response to vaccine or natural antigen and reduced memory B cells (MBCs) frequency.

Few data are available on genetic factors associated with the impaired SARS-CoV-2 response in CVID. A CVID patient with NF-kB2 loss-of-function variant who developed severe COVID-19 and a patient with TBK1 and TNFRSF13B mutations and an auto-inflammatory disease with lethal COVID-19 were reported (9, 10).

Table 1 illustrates immune alterations found in patients with CVID relevant for the impaired and variable response to vaccines (2). In the majority of patients, CVID is associated with defects in late stages of B cell development. In a group of CVID patients, plasma protein profiling identified a Th1-driven immune dysregulation, with increased plasma levels of IFN-γ and of proteins regulated by IFN-γ, NF-kB2 and NF-kB1 haploinsufficiency, or increased plasma levels of CXCL13 reflecting aberrant germinal center (GC) formation (12).

Aberrant germinal center reactions are also observed in previously healthy individuals affected by severe COVID-19. In these cases, the excessive reaction of the innate immune system with production of high concentrations of inflammatory cytokines disrupts the architecture of the germinal center thus preventing the response (13). The impaired germinal center reaction in COVID-19 leads to the generation of extrafollicular responses and increase of atypical memory B cells (14).

Thus, the inability to perform the germinal center reaction genetically determined in CVID, is induced by the explosive innate immune reaction to SARS-CoV-2 in severe COVD-19.

VACCINATIONS IN CVID

As stated above, immunization can be used in patients with impaired and residual B-cell function to provide information on specific humoral immunity and to improve the outcomes related to vaccine-preventable disease. In CVID patients, vaccination is a tool to evaluate T-dependent and T-independent antibody residual function of B lymphocytes and it might be used to

TABLE 1 | Immune alterations relevant to impaired response to vaccines in CVID.

Immune function	Abnormality
Immunoglobulin levels	Reduced or absent
Bone marrow plasma cells	Depleted
Germinal Centre reaction	Impaired
Stimulation *via* Toll like receptors 7, 8, and 9 in B cells and/or plasmacytoid dendritic cells	Impaired
Switched memory B cells	Reduced or absent
Circulating CD4 T cells	Reduced
Naive CD4 T cells	Reduced
Proliferation and activation of antigen-specific T cells	Impaired
CD40L expression	Reduced
T follicular helper cells	Decreased
T-cell receptor repertoires	Restricted
IL-2, IL-10	Reduced
IL-6	Increased
Thymic maturation	Impaired
Monocyte/dendritic cell	Defective function
Innate immune responses	Impaired function
NK cells	Reduced

Bonilla et al., 2016 (11).

measure specific cellular immunity (15). Thus, vaccination has both a therapeutic and diagnostic role, but might be also useful to predict the prognosis (16). In fact, the inability to mount a response against the pneumococcal polysaccharide antigens or the inability to maintain the antibody response over time identified CVID patients with a severe immunological impairment. These patients have a great risk of comorbidities and poor prognosis. Alternative or complementary measurements of other polysaccharide responses have also been proposed, in an attempt to increase the diagnostic accuracy. For example, responses to the less frequently used *Salmonella typhi* pure polysaccharide vaccine (Typhim Vi) have been studied. In a multicenter study, Guevara-Hoyer et al. (17) demonstrated a lack of response in both Typhim Vi and pneumococcal immunization in a group of CVID patients, suggesting that the evaluation of the specific antibody response to Typhim Vi vaccine may add clinical value to the diagnosis of impaired anti-polysaccharide antibody production in CVID.

More detailed information is available on immune responses to vaccines against viral infections, including influenza and VZV vaccination.

Influenza vaccines provide protection by generating high-affinity antibodies against viral hemagglutinin. High-affinity antibodies are produced during the germinal center (GC) reaction through the interaction between T follicular helper cells and B cells. The response to vaccination can be measured by the increase of hemagglutination-inhibiting antibodies and specific T-cell responses, demonstrated by the presence of vaccine-induced CD4 T cells and cytokine production. CVID patients with switched MBCs (Euroclass smB+) (18) were shown to have T-cell cytokine responses to vaccines comparable to those of healthy controls, but the vaccine specific antibody responses were found impaired in the group of patients with a more severe B-cell defect. As an alternative readout for the effective T-cell response to vaccination, it is possible to identify antigen-specific T cells by evaluating the upregulation of CD25 and CD134 (OX40) following *in vitro* re-stimulation with vaccine-derived peptides (19). VZV is the only human herpesvirus for which highly effective vaccines are available. The recent development of a liposome-based (HZ/su) subunit vaccine, which contains VZV, glycoprotein E and the adjuvant ASO1B, promises to change the perspectives for immunization against herpes zoster and its complications in adult CVID patients for whom a live-attenuated vaccine is not recommended (20). There is no data on the efficacy in primary antibody defects at present, but it is possible to hypothesize that, in response to the adjuvated vaccine, specific T cells might be able to undergo activation and terminal differentiation, thus envisioning a potential benefit of subunit vaccination.

INSIGHT ON SARS-CoV-2

The Adaptive Immune Responses

Effective vaccines against SARS-CoV-2 are being administered worldwide with the aim of terminating the COVID-19 pandemics. As for all immunizations, the efficacy has been linked to the production of specific antibodies, which increase in response to all vaccines in use (21). It should be underlined that in the study population of the pre-approval studies, no patients with primary immune deficiencies were included (11). In immunocompetent subjects, the level of neutralizing antibodies is highly predictive of immune protection, and mRNA vaccination generated robust, multi-component humoral and cellular immune memory to SARS-CoV-2 for at least 6 months after mRNA vaccination (22). Moreover, boosting of pre-existing immunity with mRNA vaccines in SARS-CoV-2 recovered individuals increased antibody responses (23). While immunization shots and subsequent boosters raised antibody levels in immunocompetent subjects, it is unclear which CVID patients might reach protection, have a reduced infectious risk and disease severity. Data on immunogenicity of SARS-CoV-2 vaccine in patients with CVID are still limited. Since vaccination became available, Italian CVID patients, as well as CVID from other countries, were immunized mostly with mRNA COVID-19 vaccines. Four groups have recently reported immune responses to vaccines in patients with inborn errors of immunity, including CVID patients (24–27). In the study by Hagin et al., 70% of the adult patients with predominantly antibody deficiency developed specific humoral and T-cell responses after 2 doses of SARS-CoV-2 mRNA vaccine. About one third of CVID patients did not produce Spike-specific IgG, mainly including patients with low B cell number and reduced switched memory B cells. Two thirds of the patients had produced specific antibodies, in particular those belonging to the group with normal frequency of switched memory B cells (Euroclass smB+). The authors concluded that the GC reaction had a crucial role on protective antibody generation (24). Similarly, in the paper from Romano et al, 4 CVID patients developed neutralizing antibodies against SARS-CoV-2. The only patient who failed to have a substantial rise in post vaccination titers had a marked decrease in the frequency of circulating B cells (25). In a recent paper, a high percentage of fully immunized CVID patients developed anti-Spike IgG, at significantly lower levels than in the healthy control group (26). Our data (27) in CVID patients immunized with BNT162b2 SARS-CoV-2 mRNA vaccine contrasted with the high frequency of response reported in the cohorts illustrated above. We show that only 20% of CVID patients developed both anti-Spike IgG and IgA antibodies, and one patient responded with IgG only. Moreover, the level of antibodies in the few patients who produced specific IgG and IgA was significantly lower compared to vaccinated healthy donors. CVID patients who did not mount a detectable antibody response after immunization had lower frequency of switched memory B cells and lower serum IgA and IgM levels. In detail, 10% of those with low frequencies of switched memory B cells (<2%) showed a detectable humoral response. However, similarly to what observed in immunocompetent individuals, in some CVID patients who were previously infected with SARS-CoV-2, the IgG response was boosted by the subsequent immunization, showing that SARS-CoV-2 infection might effectively prime the immune response (28). Administration of BNT162b2

SARS-CoV-2 mRNA vaccine does not induce the production of specific SIgA in the mucosa of the respiratory tract in healthy individuals (29). It is therefore unlikely that vaccination may induce mucosal protection in CVID patients lacking SIgA. In order to protect SARS-CoV-2 CVID infected patient, passively infused SARS-CoV-2 monoclonal antibodies treatment was effective and well-tolerated in patients with Primary Antibody Defects (30).

The huge variation among the response rates reported (2) confirms that CVID includes a complex mixture of patients with different levels of immune impairment. In addition, as hypogammaglobulinemia is the hallmark of CVID, we still ignore whether the response to COVID vaccines will represent a durable or transient change of the B-cell repertoire in this category of patients. Whereas we expect that after the initial decline, antibody levels will reach a plateau in control subjects reflecting the establishment of the long-lived memory plasma cell pool, this may never happen in CVID and antibodies may rapidly become undetectable. For this reason, although antibody measurement, month after vaccination, is neither indicated nor useful for the majority of vaccinees, the change in the concentration of Spike specific antibodies may represent an important measure of the ability of CVID patients to prevent SARS-CoV-2 severe disease.

Additional information can be obtained by combining the analysis of the B cell phenotype with the detection of Spike and RBD-specific B cells. We concentrated our study on the B cell response, because, independently of whether in each single patient CVID is caused by defects of B, T or innate cells, the final outcome is the lack of antibody responses measurable by immunoglobulin concentration and B cell phenotyping. High specificity and affinity are the most important characteristics of protective MBCs, generated by the adaptive immune system in response to infection or vaccination (31). While the natural course of COVID-19 is primarily characterized by the function of the innate immune system, with a secondary involvement of T and B cells, SARS-CoV2 vaccines are designed to force the adaptive immune system to generate neutralizing antibodies and Spike antigen-specific memory B and T cells. Immunocompetent subjects responded to SARS-CoV-2 immunization by generating classical MBCs with high and low binding capacity for Spike and activated MBCs (32). Moreover, they also generated few ATM B cells with low binding capacity and plasmablasts with low and high binding capacity. In CVID, impaired differentiation of mature post-GC B-cells, with severely reduction of switched MBCs and plasmablast/plasma cells are the most consistent defects. Impaired maturation of B-cells might occur also at the pre-GC stage, leading to a strongly reduced number of B cells in the periphery (33). Upon vaccination, CVID patients did not generate classical and activated MBCs, but SARS-CoV-2 vaccination induced only ATM B cells with low binding capacity to Spike protein (27). It has been suggested that ATM B cells are short-lived activated cells, in the process of differentiating into plasma cells. ATM B cells may be produced by extra-follicular reactions or failure of the GC reactions (34). High affinity plasmablasts were not produced in CVID, while one third of the patients generated low affinity plasmablasts. None of the CVID patients generated memory B cells specific for the receptor binding domain (RBD) of SARS-CoV-2, indicating the incapability of CVID B-cells to undergo somatic mutation and affinity maturation in the GC indispensable for the production of neutralizing antibodies. This impairment, associated to the generation of atypical memory or classical memory B cells with low affinity for the Spike protein is the basis to hypothesize a sub-optimal and transient humoral immune response after vaccination in CVID patients.

Remarkably, CVID patient convalescents from COVID-19 generated classical Spike-specific MBCs after vaccination (28). Since natural infection responses are boosted by subsequent immunization, the comparison of immune responses generated by the vaccine and the infection will be important to shed light on the difference between an antigen-driven response and an infection-driven response.

The role of T cells in antiviral responses and formation of immunological memory in general is well-recognized. SARS-CoV-2-specific T cells have been identified in all T cell subsets, TCM, TEM, and TEMRA subsets in immunocompetent individuals. Indeed, several studies have demonstrated T cell memory and effector responses against a broad selection of epitopes from SARS-CoV-2, as well as cross-reactive responses in unexposed individuals (35). The majority of the immunocompetent subjects developed T-cell responses following immunization, with variable results observed only in aged individuals (36).

Spike-specific T-cells responses were induced in CVID patients with a variable frequency (37, 38), differently from T cells produced after multiple exposures to influenza viral antigen following immunization or infection. In a recent study, the vast majority of CVID patients had S1-specific T cells compared after two doses of immunization with mRNA vaccine (39). In our study (27), differently from influenza immunization, poor Spike-specific T-cell responses were generated by immunization. SARS-CoV-2 is a pathogen never encountered before, since SARS-CoV-2 Spike and the RBD domains are distinct from the Spike proteins of most members of the coronavirus family (40). Then, it is possible that the first antigenic stimulation was not sufficient to induce an early T-cell response in CVID.

The Innate Immunity Responses

Cells of the innate immune system play an essential role in early protection against infectious disease. To pathogens for which there is no preexisting immunity, the innate immune system is activated with the intent of limiting the infection while the adaptive immune response develops slowly and needs two weeks to generate the most specific and effective defensive tools: high affinity antibodies and memory B and T cells (41). After two years from the beginning of the pandemic, it remains unclear whether the condition of primary antibody deficiency is a predisposing or a protective factor for SARS-CoV-2 infection (42). Moreover, treatments for the immune deficiency status might also interfere with the disease progression and to the response to SARS-CoV-2 immunization. It should be

remembered here that patients with antibody deficiencies receive monthly immunoglobulin replacement therapy to substitute the lack of antibody production. The pool of immunoglobulin might include cross-reacting antibodies to SARS-CoV-2, and might act by modulating monocytes and macrophages activities, even when administered at replacement dosages (43). We suggested (44) that antibody deficiency patients might be protected from severe COVID-19 by loss of Interleukin-6 and by impaired toll-like receptor (TLR) pathway activation. TLR pathway activation is impaired in CVID, particularly activation by TLR7 and TLR9, involved in antiviral innate immune responses and in the cytokine storm leading to an exaggerated activation of lung residing immune cells (45). Thus, CVID patients might be partially protected against the dangerous macrophage hyperactivation resulting in cytokine storm. On the contrary, CVID patients with an underlying inflammatory chronic lung disease have a worse COVID-19 prognosis (46). CVID-associated immune dysregulation is a Th1-mediated inflammatory process driven by the IFN-γ pathway (47) and by a persistent activation on innate immunity (48), possibly due to the activation of IFN-γ:STAT1:BAFF axis leading to a dysregulated B cell responses (49). The interferon signature in CVID has been also linked to the expansion of circulating IFN-γ-producing innate lymphoid cells (50). Differently from COVID-19, the highly augmented IFN signaling and cytotoxic signature has not been detected after vaccination with the SARS-CoV-2 mRNA vaccines (51). In particular, the upregulation of gene signature associated with type I and type II IFN production was not observed in the immunized subjects, suggesting an adaptive immunity maturation in the absence of IFN signaling.

CONCLUSIONS

Despite the antibody deficiency, T-cell immunity is thought to be largely intact in many patients with CVID. For this reason, immunologists recommend routine administration of multiple vaccines with the exception of those containing attenuated viruses. Immunization of CVID patients against SARS-CoV-2 offered the possibility to analyze how defective mechanisms impact the immune response to a novel antigen. Discrepancy in the results published on antibody responses after SARS-CoV-2 immunization in CVID might be due to the heterogeneity of CVID populations enrolled or to different vaccination protocols. In CVID as well as in immunocompetent subjects, the nature of B- and T-cell responses differs dramatically between infected and vaccinated individuals, suggesting that inflammatory responses associated with infection influence the trajectory of the adaptive immune response. Moreover, the observation that the humoral immune response induced by natural infection was significantly enhanced by subsequent immunization underlines the need to immunize COVID-19 convalescent CVID patients after recovery. The search for a strategy to elicit an adequate protective immune response post-vaccination in CVID patients is necessary. This might include the changes of vaccination schedules, such as administration of multiple doses or to booster with heterologous vaccine preparation. In order to achieve protection of patients with immunodeficiency, new strategies may be necessary, such as the development of different types of vaccines, including those with inactivated viruses, high content of antigens, or with adjuvant. At present, we do not know whether CVID patients might require multiple doses or combinations of SARS-CoV-2 vaccines to obtain protection. Since reinfection with SARS-CoV-2 after vaccination or previous infection has been documented in CVID, at present SARS-CoV-2 positive CVID patients might benefit from the new preventing strategy based on administration of anti-SARS-CoV-2 monoclonal antibodies, and - in the next future - by the newly produced lots of gamma globulins for substitution therapy that will contain Spike-specific IgG.

The results obtained by the administration of mRNA vaccines against a virus never encountered by humans before has given us the possibility to study the different immune responses of the complex community of CVID patients and compare them to healthy controls. Thanks to the availability of new tools and methods, developed because of the COVID-19 pandemic, we are able not only to measure antibodies but also antigen-specific B and T cells. We should now combine the results obtained with the complete vaccine cycle and the booster dose, completed by a follow-up to measure the persistence of specific antibody and memory B cells. On the basis of this study it will be possible to distinguish the group of CVID patients who may benefit from vaccination and also pin-point the specific step of the immune response defective in each individual.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

IQ and RC elaborated data and wrote the manuscript. FL revised and commented the manuscript. All authors defined the conclusion. All authors contributed to the article and approved the submitted version.

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Inborn Errors of Immunity and Their Phenocopies: CTLA4 and PD-1

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Elucidating links between genotype and phenotype in patients with rare inborn errors of immunity (IEIs) provides insights into mechanisms of immune regulation. In many autosomal dominant IEIs, however, variation in expressivity and penetrance result in complex genotype-phenotype relations, while some autosomal recessive IEIs are so rare that it is difficult to draw firm conclusions. Phenocopies arise when an environmental or non-genetic factor replicates a phenotype conferred by a specific genotype. Phenocopies can result from therapeutic antibodies or autoantibodies that target a protein to replicate aspects of the phenotype conferred by mutations in the gene encoding the same protein. Here, we consider IEIs arising from rare genetic variants in *CTLA4* and *PDCD1* and compare clinical and laboratory manifestations arising as drug-induced phenocopies (immune related adverse events, IRAEs) in cancer patients treated with immune checkpoint inhibitors (ICI) and identify outstanding questions regarding mechanism of disease.

Keywords: CTLA4, PD-1, immune checkpoint inhibitor, phenocopy, immune deficiency

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INTRODUCTION

Autosomal dominant loss of function mutations in *CTLA4* result in a complex syndrome of immune dysregulation and deficiency (1–3), although the syndrome is characterized by variable expressivity and incomplete penetrance. Recently, human *PDCD1* deficiency was described. So far, this syndrome appears to be exceedingly rare, whereas we have extensive experience with ICI that target PD-1 or its ligand., as well as anti-CTLA4 antibodies. Comparing and contrasting these phenocopies with IEIs of *CTLA4* and *PDCD1* might advance our understanding of the actions of CTLA4 and PD-1, and how defective expression of these molecules cause immune deficiency and dysregulation (4).

CTLA4

CTLA4 is a transmembrane receptor that is structurally similar to CD28 but acts as an inhibitor of T cell activation (5–10). CTLA4 is expressed constitutively by regulatory T cells (Tregs) and is indispensable for immunological self-tolerance and immune homeostasis (11). Conventional T cells upregulate CTLA4 expression upon stimulation, mediated at least in part by nuclear factor of activated T-cells (NFAT) (12). CTLA4 expression has also been reported on B cells, fibroblasts, $CD34^+$ stem cells and granulocytes (13–15), but the significance and action of CTLA4 expression on these cells remains to be determined. CTLA4 is expressed in immune cell malignancies including leukemic B cells and also by breast cancer cells, melanoma, and various carcinomas (16–18).

There is evidence that CTLA4 acts in several ways to modify T cell activation. CTLA4 inhibits co-stimulation by outcompeting CD28 for CD80/86 (19), and real-time competition between CTLA4 and CD28 for translocation into the central-supramolecular activation clusters (cSMAC) of immune synapses has been demonstrated (20). CTLA4 has also been reported to recruit protein phosphatase 2A (PP2A) and tyrosine phosphatase SHP-2 *via* its cytoplasmic tail, which then dephosphorylates many kinases including AKT, ERK and MEK to inhibit T cell activation (21–23). This cell-intrinsic action has been challenged, however, by reports of a mouse model expressing mutant CTLA4 lacking a cytoplasmic tail, which has no lymphocytic infiltrates or autoimmune disease (24).

Other evidence suggests that CTLA4 acts cell-extrinsically to modify immunity by reducing availability of CD80 and CD86. CTLA4 is a highly endocytic molecule and has been shown to capture and remove CD80/86 from antigen presenting cells, directing these ligands to lysosomes for degradation (25). Other possible actions include regulation of cell adhesion and motility. Ligation of CTLA4 has been postulated to increase T cell motility and reduce contact periods between T cells and antigen-presenting cells, which could prevent inappropriate activation of T cells with low-affinity for peptide-MHC complexes (26). Ligation of CTLA4 recruits PKC-eta, which forms a complex with GIT-2 and PAK-2 to modulate Treg cell-APC interactions. Consistent with this, PKC-eta deficient Tregs fail to dissociate from APCs and exhibit a defect in CD86 capture and transendocytosis (27).

CTLA4 HAPLOINSUFFICIENCY

CTLA4 haploinsufficiency (abbreviated here as *CTLA4*$^{+/-}$) leads to a syndrome of immune dysregulation with a broad spectrum of clinical manifestations, and in approximately 30% of carriers, no clinical manifestations at all (1–3). In the largest cohort described to date, the median age of disease onset was 11 years, with a range of 1-59 years. Thus, in many cases, onset of clinical manifestations is not observed until adulthood. Of the clinical phenotypes, lymphoproliferation occurs frequently (73%). Autoimmune and inflammatory manifestations are also common, although there is considerable variability in the end-organs affected. Lymphocytic infiltration of lung, gastrointestinal tract, brain, bone marrow, kidney and retroperitoneal tissue have all been reported. Hematological cytopenia (immune thrombocytopenic purpura, ITP; and autoimmune hemolytic anemia, AIHA) are frequent, while atrophic gastritis, coeliac disease and pancreatitis are uncommon (1–3) (**Table 1**).

Respiratory manifestations are common in *CTLA4*$^{+/-}$ patients. In addition to lymphocytic pneumonitis, many *CTLA4*$^{+/-}$ patients have recurrent respiratory tract infections, including pneumonia, sinusitis and otitis media. Infective complications are accompanied by hypogammaglobulinemia (84%), including reduced IgA (40%), IgG (32%) and IgM (30%) (1–3) (**Table 1**).

CTLA4$^{+/-}$ patients have hyperactivated effector T cells with increased expression of PD-1 and HLA-DR. In some studies, CD4$^+$ FoxP3$^+$ Treg cells have been reported to be increased (1, 3) but this has not been a consistent finding (2). B cell abnormalities in *CTLA4*$^{+/-}$ patients include reductions in switched memory B cells, progressive loss of all peripheral B cells, and increased CD21^{low} B cells (1–3). Interestingly, despite the hyperproliferation, T and B cells undergo increased apoptosis *in vitro* (2).

In the mouse model, *Ctla4* haploinsufficiency has not been reported to cause a phenotype but *Ctla4*$^{-/-}$ mice develop a lethal lymphoproliferative disorder by 3-4 weeks of age. Mice exhibit progressive skewing of the T cell compartment towards CD4$^+$ T cells, lymphocytic infiltrates occur in multiple organs, and pathology is prevented by CD4$^+$ T cell depletion (38). When *Ctla4* deficiency is confined to Tregs, mice exhibit delayed lymphoproliferation and fatal T cell-mediated autoimmune disease (including pulmonary lymphocytic infiltrates) by 7 weeks of age (11). T cells are activated with upregulation of activation markers CD44, CD69 and CD25 (9, 10). *Ctla4*$^{-/-}$ mice also exhibit

TABLE 1 | Inborn errors of CTLA4 and their phenocopies.

	CTLA4 haploinsufficiency				**CTLA4 checkpoint inhibitor**			
Main clinical phenotypes		%	Immune/histological phenotype	Ref.	All (%)	Grade 3-5 (%)	Immune/histological phenotype	Ref.
	Hypogammaglobulinemia	84%	Expanded T cells with upregulated of activation markers. Increased IL-4 and IFN-γ-producing CD4$^+$ T cells. Impaired suppressive function by Tregs. Lymphocytic infiltration in multiple organs.	(1–3)	ND		Dermatologic: Skin T cells infiltrate.	
	Lymphoproliferation	73%			ND		Gastrointestinal: Enterocolitis with neutrophilic, lymphocytic infiltrate and both.	(28–30)
	Respiratory tract manifestation	57-68%			1-11%	1-2.7%		
	Autoimmune cytopenia	62%			<1%	<1%	Hypophysitis: Autoantibodies against TSH, FSH and ACTH-secreting cells.	(31)
	Gastrointestinal manifestation	59%			30-40%	7.6-17%		(32–34)
	Dermatologic manifestation	21-56%			44-59%	1-4%		(32, 33, 35, 36)
	Thyroiditis/ Hypothyroidism	14%			1.5-9%	0-1%		(32, 33)
	Liver manifestation	12%			3.8-8%	1%		(29, 32, 33)
	Hypophysitis	1%			13%	5%		(33, 37)

ND, Not Detectable.

macrophage and neutrophil infiltration of end-organs, including heart, lung, salivary glands, liver, bone marrow and pancreas. Interestingly, immunodeficiency observed in humans with *CTLA4* haploinsufficiency is not observed in *Ctla4*$^{-/-}$ mice.

PHENOCOPIES OF *CTLA4* DEFICIENCY

In the 1990s, Allison and colleagues identified the therapeutic potential of CTLA4 inhibition, which culminated in the development and use of ICIs targeting CTLA4 for cancer therapy (39, 40). Ipilimumab and tremelimumab bind to the same region of CTLA4 and interfere with CD80/86 recognition (41–43). Early clinical trials reported that anti-CTLA4 provided durable clinical responses and improved overall survival in a fraction of cancer patients (32, 44). ICIs are now standard of care for many forms of cancer (45). Autoimmunity and inflammatory side effects, however, emerged as significant complications in a proportion of treated patients. Severe IRAEs (Common terminology criteria for adverse events (CTCAE) severity grade of 3-5) have been reported in 19.9-24% of melanoma patients treated with ipilimumab and 52% of melanoma patients treated with tremelimumab (28, 29, 32, 35). The most common severe IRAEs are colitis, dermatitis, and endocrinopathies of hypophysitis and hypothyroidism. Hepatotoxicity, hematological cytopenia and neurologic complications are also observed but are less frequent (31–33) (**Table 1**).

Colitis of any grade, which most commonly presents with diarrhea, has been reported in 30-40% of patients treated with ipilimumab, while severe colitis/diarrhea is seen in up to 7.6-17% patients on anti-CTLA4 treatment (32, 33) (**Table 1**). Three histological types of enterocolitis are described: neutrophilic (46%), lymphocytic (15%), and combined neutrophilic and lymphocytic (38%). Neutrophilic inflammation is mainly associated with cryptitis, while lymphocytic inflammation is characterized by increased $CD8^+$ T cells in the crypt epithelium and $CD4^+$ T cells in the lamina propria (34). Gastrointestinal involvement is also common with *CTLA4* haploinsufficiency and histology usually reveals extensive T cells infiltration (1–3).

Severe hypophysitis is observed in 5% of melanoma patients treated with ipilimumab but is rare in *CTLA4*$^{+/-}$ patients (1 of 133) (3, 33) (**Table 1**). Repeated injection of anti-CTLA4 antibody results in pituitary infiltration of lymphocytes, macrophages and monocytes. In addition, pituitary autoantibodies are detected in mice and melanoma patients after injection of anti-CTLA4 antibody (37). Severe hypothyroidism has been reported in melanoma patients with anti-CTLA4 treatment but is uncommon (~1%) (33). Autoimmune thyroiditis appears to be more frequent in *CTLA4* haploinsufficiency (18 of 133) (3). Furthermore, common variants affecting the *CTLA4* promoter (49A/G or 60C/T) segregate with autoimmune hypothyroidism (46, 47).

Severe pneumonitis is observed in about 1-2% of patients treated with ipilimumab (28, 29) and bronchospasm has been reported after tremelimumab treatment (2.7%) (30). Respiratory symptoms have been reported in 57-68% patients in different studies. As noted above, many *CTLA4*$^{+/-}$ patients also suffer from recurrent respiratory tract infections but has been reported infrequently as a complication of anti-CTLA4 treatment (48) (**Table 1**).

Skin-related IRAEs are common and generally mild in patients receiving anti-CTLA4 treatment. Severe dermatological IRAEs, including pruritus, rash and vitiligo, are observed in up to 4% of patients treated with either ipilimumab or tremelimumab (32, 33, 35, 36). Histological analysis has revealed perivascular immune cell infiltrates in the dermis and epidermis. Although both $CD4^+$ and $CD8^+$ T cells are identified on biopsy of macules, $CD4^+$ T cells dominate the infiltrates reported in melanoma patients receiving ipilimumab (49–51). CTLA4 blockade has also been reported to increase epidermal thickness and infiltrating T cell counts in mice with psoriasis (52). Psoriasis, atopic dermatitis and vitiligo have been observed in in 21-56% of *CTLA4*$^{+/-}$ patients (1, 3) (**Table 1**).

Hepatitis has been reported in 3.8-8% of patients receiving ipilimumab but severe hepatic toxicity occurs in less than 1% of patients (29, 32, 33). Liver involvement with lymphocytic infiltrate and liver failure has been observed in 12% of *CTLA4*$^{+/-}$ patients (1, 3).

COMPARISON OF CTLA4 DEFICIENCY AND ITS PHENOCOPY

While the nature and spectrum of IRAEs after anti-CTLA4 treatment is similar to the autoimmune and inflammatory complications of *CTLA4* haploinsufficiency, these manifestations appear to be more common in *CTLA4* haploinsufficiency. This may reflect the extent of CTLA4 blockade. Consistent with this proposition, IRAEs related to ipilimumab are dose-dependent. Serious IRAEs are more common with higher doses of ipilimumab, mainly due to increased adverse events in gastrointestinal tract, skin and endocrine organs (53). Similarly, low-dose anti-CTLA4 antibody treatment in mice induces anti-parietal autoantibodies, high-dose anti-CTLA4 antibody infection leads to histologically evidence of autoimmunity (54).

Remarkably, hypophysitis is considerably more prevalent in patients treated with anti-CTLA4 antibodies than in patients with *CTLA4* haploinsufficiency. CTLA4 is expressed by both human and mice non-hematopoietic cells in the pituitary gland, and anti-CTLA4 antibodies bind to these cells. CTLA4 is also expressed by pituitary cells, particularly those responsible for secreting prolactin and TSH (37).

Differences in Fc receptor binding by ICIs have been investigated for their contributions to therapeutic actions but might also contribute to differences in IRAEs when compared with CTLA4 haploinsufficiency (55). For example, ipilimumab and tremelimumab are IgG1 and IgG2 antibodies, respectively. IgG1 binds to multiple FcRs whereas IgG2 is thought to bind to FcγRIIB and the H131 isoform of FcγRIIA. Different subclasses might also account for the longer half of tremelimumab (22 days) relative to ipilimumab (14 days) (41, 43). Different Fc components might explain other differences as well. Mouse

studies have shown FcR binding results in Treg depletion by interaction with tumour-infiltrating myeloid cells, which is crucial for their anti-tumour effects (56, 57). By contrast, *CTLA4* haploinsufficiency results in deficiency of ligand binding independently of FcR ligation, which might result in differences in Treg depletion and other effects outside of the tumour environment. Differences in FcR-mediated actions might also identify pathology that is predominantly antibody-mediated. For example, hypophysitis is thought to result from complement activation by C1q binding to the Fc fragment of anti-CTLA4 antibody (37).

One major discrepancy between immune disorders in patients with *CTLA4* haploinsufficiency and those receiving anti-CTLA4 treatment is the increased susceptibility to infection in *CTLA4*$^{+/-}$ patients. Most *CTLA4*$^{+/-}$ patients present with recurrent respiratory tract infections, which is thought to result from deficiency of B cells and immunoglobulin (1, 3). Serious infections appear to be less frequent in patients receiving immune checkpoint therapy. In one study, they were observed in 54/740 patients (7.3%). Furthermore, the contribution of ICIs to infection is confounded by concurrent immunosuppression to manage IRAEs. In one study, serious infections were observed in 13.5% of melanoma patients treated with either corticosteroids or infliximab but in only 2% in those who did not require immunosuppression (48).

Since LRBA competes with AP-1 for the YVKM motif on the cytoplasmic tail of CTLA4 to protect CTLA4 from lysosomal degradation (58), LRBA deficiency results in reduced CTLA4 expression and could therefore be informative for understanding the *CTLA4*$^{+/-}$ phenotype. Similar to *CTLA4*$^{+/-}$ patients, *LRBA* deficiency also confers increased risk of recurrent respiratory tract infections, and most patients with homozygous or biallelic mutations in *LRBA* are also diagnosed in early childhood with hypogammaglobulinemia (57%), B cell lymphopenia, particularly affecting memory B cells and plasmablasts (58–60), while heterozygous carriers are healthy. Most *LRBA*$^{-/-}$ patients have immune dysregulation encompassing enteropathy and hematological cytopenia (AIHA and ITP). Organomegaly, including splenomegaly and lymphadenopathy, is also prevalent, while T1D and hepatitis are less common (58–60). Two groups have reported that *Lrba*$^{-/-}$ mice do not have any sign of immunological disorders, either at steady state or after challenge with virus or bacterial infection. Another group reported that *Lrba*$^{-/-}$ mice are susceptible to DSS-induced colitis, although this phenotype was suggested to arise from dysregulation of TLR signaling rather than impaired CTLA4 expression (61–63). In mice, conventional B and T cell development does not appear to be affected by LRBA deletion, although peritoneal B1-a cells are reduced.

PD-1

In the 1990s, Honjo and colleagues discovered and characterised PD-1 as a negative T cell regulator (64–67). The therapeutic potential of PD-1 blockade in cancer therapy was illustrated in *Pdcd1*$^{-/-}$ mice and then confirmed in cancer patients after anti-PD-L1 treatment (68). PD-1 is expressed by T cells, NK cells, B cells and activated monocytes (69). PD-1 expression is considered to be a marker of cell exhaustion, and PD-1^{+} cells exhibit reduced cytokine production and reduced proliferative capacity (70–72). Nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) and interferon-stimulated responsive element (ISRE) bind directly to the PD-1 promoter to upregulate PD-1 expression in response to TCR and IFN-α stimulation, respectively (73, 74), whereas T-bet and Blimp-1 suppress PD-1 expression (75, 76). Posttranslational modifications also regulate PD-1 expression, which provides potential novel avenues for PD-1-related therapy. Fucosylation of PD-1 at positions N49 and N74 by Fut8, a core fucosyltransferase, is required for cell-surface expression of PD-1 (77). Moreover, PD-1 is degraded in proteasome after Lys48-linked poly-ubiquitination by the E3 ubiquitin ligase, FBXO38. Deletion of FBXO38 leads to faster tumor progression with increased PD-1 expression in tumor-infiltrating T cells (78).

There are two PD-1 ligands. PD-L1 is expressed on T cells, B cells, dendritic cells, macrophages and non-hematopoietic cells, while PD-L2 is restricted to dendritic cells and macrophages (69). PD-L1 expression is upregulated in many tumors, including melanomas, non-small cell lung cancer and ovarian cancer (79–82). Upon IFN-γ stimulation, PD-L1 upregulation is mediated by various transcription factors, including IRF-1, MyD88, TRAF6 and MEK (83, 84). In addition, chimeric nucleophosmin (NPM) and anaplastic lymphoma kinase (ALK) induce the expression of STAT3, which upregulates PD-L1 expression (85). PD-L1 expression is also regulated post-transcriptionally. Glycogen synthase kinase 3β (GSK 3β) phosphorylates non-glycosylated PD-L1, leading to the proteasomal degradation by β-transducin repeat-containing protein (β-TrCP) (86), while COP9 signalosome 5 (CSN5) induced by NF-κB p65 (RelA) upon TNF-α stimulation inhibit ubiquitination and degradation of PD-L1 (87).

Ligation of PD-1 by PD-L1 results in transduction of a negative signal that suppresses T cell activation, cytokine production, survival and proliferation. LCK phosphorylates the immunoreceptor tyrosine-based switch motif of PD-1 cytoplasmic tail (88), recruiting Src homology region 2 domain containing phosphatase-1 (SHP-1) and SHP-2, which dephosphorylate many signaling molecules, including ZAP70/CD3zeta and ERK in TCR signaling pathway (88, 89), as well as PI3K/AKT/mTOR in CD28 signaling pathway (90–92). There is evidence that PD-1 binds preferentially to SHP-2 and dephosphorylates the CD28 cluster in the immune synapse (93). Additionally, ICOS co-stimulation of T cells for proliferation and cytokine production is also inhibited by PD-1 ligation (94). PD-1 also suppresses TCR-driven signal that stops cellular migration to increase the contact time of antigen-specific T cells with dendritic cells (70). The inhibitory function mediated by PD-1-PD-L1 ligation could also be indirect. In the absence of PD-1 ligation, TCR stimulation upregulates the expression of Ser/Thr protein kinase CK2, which stabilizes PTEN protein as a negative regulator of PI3K/Akt signaling pathway (95).

PDCD1 DEFICIENCY

Recently, a rare, homozygous frameshift mutation (c.105dupC, p.T36Hfs*70) in *PDCD1* was identified in a patient with tuberculosis and autoimmunity (96). The mutation was shown to abrogate PD-1 expression. The patient was diagnosed with type 1 diabetes (T1D), hypothyroidism and juvenile idiopathic arthritis (JIA) by the age of 3 years, developed large, multifocal intraperitoneal abscesses and abdominal tuberculosis by the age of 10 years, and died of pneumonitis one year later. Stimulated leukocytes from the patient exhibited reduced IFN-γ production. The numbers of $V\delta2^+$ γδ T cells, mucosal-associated invariant T cells, and $CD56^{bright}$ natural killer cells were decreased, but helper T cell subsets were within normal range. Interestingly, the patient exhibited hepatosplenomegaly with expanded $CD38^+$ activated and $ROR\gamma T^+$ $CD4^-$ $CD8^-$ double-negative αβ T cells, similar to the phenotype displayed by *STAT3* GOF patients, and STAT3-dependent cytokines IL-6 and IL-23 were increased (96) (**Table 2**).

About one third of $Pdcd1^{-/-}$ C57BL/6 mice develop arthritis and mild proliferative glomerulonephritis by 6 months of age, with extensive renal IgG3 and C3 deposition, and these manifestations become more frequent and severe over time (65). On a BALB/c background, only 30% of $Pdcd1^{-/-}$ mice survive to 40 weeks due to autoimmune myocarditis (66).

PHENOCOPIES OF *PDCD1* DEFICIENCY

IRAEs are less frequent in patients receiving ICIs directed against PD-1 and PD-L1 than with CTLA4 ICIs. Severe IRAEs are have been reported in up to 16.3% of melanoma patients and 10% of non-small-cell lung cancer patients treated with nivolumab (anti-PD-1) (97, 106, 107, 109, 110), 10.1-14.7% of melanoma patients and 9.5-26.6% of non-small-cell lung cancer patients treated with pembrolizumab (anti-PD-1) (28, 101–104), and 11-15% of non-small-cell lung cancer or metastatic urothelial cancer patients with atezolizumab (anti-PD-L1) (105, 111, 112). By contrast with IRAEs after ipilimumab, which appear to be dose-dependent, IRAEs with anti-PD-1/PD-L1 are independent of dose (53, 102, 113). The IRAEs related to anti-PD-1/PD-L1 treatment are mostly mild (28, 97, 105, 107, 110). Pembrolizumab and nivolumab bind to partially overlapping epitopes on PD-1, and both outcompete PD-L1 for binding to PD-1 due to their high affinity. Interestingly, both agents are human IgG4 antibodies, in which Fc regions exhibit low affinity for complement protein C1q and FcRs (114, 115).

Severe colitis has only been reported in 1-2% patients after anti-PD-1/PD-L1 treatment, which is much less frequent than after anti-CTLA4 therapy (98, 102, 105–107) (**Table 2**). Histology reveals both neutrophilic and lymphocytic inflammation (116). Respiratory IRAEs have been reported in 2.2-16% of patients after anti-PD-1/PD-L1 treatment. Severe pneumonitis was reported in 0.8-2% of patients treated with pembrolizumab (98, 101, 102, 105) (**Table 2**). Anti-PD-1/PD-L1 therapy related pneumonitis is more likely to occur in non-small cell lung cancer than melanoma and renal cell carcinoma (117). Cryptogenic organizing pneumonia is the main pattern in PD-1 inhibitor-related pneumonia, followed by nonspecific interstitial pneumonia (118).

Thyroiditis can present with either hypothyroidism or hyperthyroidism, and is observed in 8-24.5% patients after anti-PD-1/PD-L1 treatment. Severe hypothyroidism or hyperthyroidism occurs in less than 1% (28, 98, 101–105). Interestingly, individuals with pre-existing anti-thyroid autoantibodies are significantly more susceptible to thyroid IRAEs induced by PD-1 inhibitor (119, 120). T1D and adrenal insufficiency have been observed in about 1% of patients with pembrolizumab treatment (28, 101, 104) (**Table 2**).

Rash, pruritus and vitiligo occur in 16-50% of patients, while severe skin disorders occur in less than 3% (28, 97, 98) (**Table 2**). Patients with pre-existing autoantibodies and rheumatoid factor are more susceptible to skin IRAEs (99). In patients with pre-existing psoriasis, anti-PD-1 treatment has been shown to

TABLE 2 | Inborn errors of PDCD1 and their phenocopies.

	Genetic PD-1 deficiency				**PD-1 or PD-L1 immune checkpoint inhibitor**			
Main clinical phenotypes		%	Immune/histological phenotype	Ref.	All (%)	Grade 3-5(%)	Immune/histological phenotype	Ref.
	Dermatologic manifestation	Present*	Impaired IFN-γ production by T cells.	(96)	16-50%	<3%	Dermatologic: Increased serum IL-6 and skin CD8/CD4 ratio.	(28, 97–100)
	Thyroid diseases	Present			8-24.5%	<1%		(28, 98, 101–105)
	Gastrointestinal manifestation	Not described	Decreased Vδ2+ γδ T cells, mucosal-associated invariant T cells, and CD56bright natural killer cells.		1-20%	1-2%	Gastrointestinal: Neutrophilic and lymphocytic inflammation. Liver: lymphocyte infiltration and rare plasma cell and eosinophil.	(98, 102, 105–107)
	Respiratory tract manifestation	Present			2.2-16%	1-2%		(98, 101, 102, 105)
	Liver manifestation	Present			5.2-18%	<1%		(98, 103, 108)
	Type 1 diabetes	Present			<1%	<1%		(28, 101, 104)
	Tuberculosis	Present	Increased CD4- CD8- double-negative αβ T cells.		–			–

** - Only one child with inherited PD-1 deficiency is described yet.*

increase $CD8^+/CD4^+$ T cells ratio of infiltrating skin lymphocytes. The level of IL-6 but not IL-17A, IFN-γ and IL-8 in serum is significantly increased in cancer patients developing psoriasis-like dermatitis after anti-PD-1 treatment (100). Severe transaminitis occurs in 1% of patients after anti-PD-1 treatment (98, 103). Histology analysis indicates most patients exhibit the lobular inflammation with lymphocytic infiltrate and rare infiltration of plasma cells and eosinophils (108).

COMPARISON OF *PDCD1* DEFICIENCY AND ITS PHENOCOPIES

The spectrum and manifestations of IEIs of *PDCD1* and phenocopies arising as IRAEs after PD-1 inhibitors overlap. *PDCD1* deficiency (c.105dupC, T36Hfs*70) resulted in T1D and hypothyroidism at the age of 3 years, rash and stomatitis at the age of 11 years (96). A proteome-wide serum autoantibody profile revealed antibodies related to autoimmune thyroiditis and T1D (96).

Experimental models provide insight into these complications. First, PD-1-PD-L1 is critical to maintain intestinal tolerance and prevent experimental autoimmune enteritis. In a transgenic mouse model in which ovalbumin (OVA) was expressed as a neo-self-antigen by intestinal epithelial cells, either PD-L1 deletion and blockade resulted in significant weight loss and intestinal inflammation in mice transferred with OVA-specific $CD8^+$ T cells (121). Similarly, in a model of intestinal injury, *PD-L1*$^{-/-}$ mice exhibited increased mortality and weight loss, diarrhea and rectal bleeding. PD-L1 expression on non-hematopoietic intestinal parenchyma prevented TNF-α production and conferred protection from intestinal inflammation. Interestingly, *PD-L1*$^{-/-}$ *Rag*$^{-/-}$ mice have a significantly higher death rate and morbidity than *Rag*$^{-/-}$ mice, indicating a contribution by innate immunity (122). Interestingly, however, gastrointestinal abnormalities were not reported in the patient with *PDCD1* deficiency (96).

By contrast, destructive thyroiditis was observed in both the *PDCD1*-deficient patient and in cancer patients treated with anti-PD-1 antibodies. Furthermore, thyroid infiltration of $PD\text{-}1^+$ T cells is observed in sporadic Graves' disease (123), and a mouse thyroiditis model induced by thyroglobulin immunization is exaggerated by anti-PD-1 treatment, which is prevented bydeletion of $CD4^+$ T cells. In this model, thyroid infiltrating $CD4^+$ T cells acquire cytotoxic features and potentially kill thyrocytes *via* specific recognition of thyroglobulin antigen (120).

T1D is observed in cancer patients after anti-PD-1 treatment and occurred in the *PDCD1*- deficient patient. A 7146G/A polymorphism in *PDCD1* gene has been reported to confer significantly increased susceptibility to T1D (124). T1D is accelerated and completely penetrant after PD-1 deletion in NOD mice, which appears to result in enhanced T cells infiltration of β-islets, with increased IFN-γ production (125) although autoantibodies against insulin were not increased compared with WT NOD mice (125). Deficiencies of *PD-L1*, *PD-L*$^-$ and *PD-L1* have all been shown to accelerate development of diabetes in NOD mice. Pancreatic lymph nodes from *Pdl1*$^{-/-}$ *Pdl2*$^{-/-}$ NOD mice also have more IFN-γ and TNF-α producing T cells. Interestingly, PD-L1/PD-L2 expression on nonlymphoid cells is sufficient to control the progression of autoimmune diabetes (126).

Finally, the *PDCD1* patient had dermatitis (96). Consistently, mice with *Pdcd1* deletion on $CD8^+$ T cells are more susceptible to psoriasis-like dermatitis induced by imiquimod (R848, a toll-like receptor 7/8 agonist), which is ameliorated by anti-IL-6 receptor blockade (100). In another contact hypersensitivity mouse model induced by hapten, PD-1 deletion and anti-PD-L1 treatment also lead to enhanced skin infiltration by $CD8^+$ T cells (127).

CONCLUSIONS

IRAEs observed in patients treated with antibodies targeted at CTLA4 and PD-1 and the receptors of their ligands phenocopy the autoimmune manifestations of patients with IEIs of *CTLA4* and *PDCD1*. There are differences in the spectrum of autoimmune manifestations between IEIs and IRAEs, such as hypophysitis. ICIs phenocopy defects in ligand binding, but also result in FcR ligation. This difference merits further investigation as a possible cause of phenotypic discrepancies between IEIs and their phenocopies. Another important observation, however, is that analysis of IEIs and their therapeutic phenocopies suggest that humans are more dependent on checkpoint inhibition than mice for protection against autoimmunity and inflammation.

Clinical manifestations arise in the majority of patients with *CTLA4* haploinsufficiency. By contrast, lymphocytic pathology arises only in mice homozygous for *Ctla4* deletion. This suggests that in addition to the genetic defect in *CTLA4* in its IEI, or after CTLA4 blockade, additional factors may promote inflammatory pathology in humans that do not act in the mouse model, at least not in the specific pathogen free environments in which experimental mice are maintained. This conclusion is supported by observations with IEIs arising from *LRBA* deficiency, which results in a reduction in CTLA4 expression. LRBA deficiency often results limited autoimmunity in humans, whereas *Lrba*-deficient mice are either healthy, or exhibit inflammation only after substantial environmental stress (e.g. DSS administration), which might provide further evidence that humans are more sensitive to changes in CTLA4 expression than mice.

The other significant phenotypic discrepancy is immune deficiency. These are common in IEI affecting CTLA4, but are not observed as IRAEs, nor do they feature in mouse models of *Ctla4* deficiency. *LRBA* mutations also result in hypogammaglobulinaemia and B cell deficiencies. Based on the distribution of CTLA4, LRBA and PD-1 expression, the effect is unlikely to be cell-intrinsic to the B cell compartment, but this will need to be resolved empirically. In some patients, $CD21^{low}$ B cells, which are said to be exhausted, have been observed to be expanded in patients with *CTLA4* deficiency, and this population has been simultaneously used to explain the hypogammaglobulinemia, and the increased incidence of autoantibodies (128, 129). While antibody deficiency has been observed in humans with *LRBA* deficiency, and this might reinforce the evidence that *CTLA4* deficiency causes antibody

deficiency, other potential mechanisms, most notably B cell intrinsic defects in autophagy could explain this phenotype.

Further work will be required to resolve this fascinating discrepancy. Possible contributions include the age of onset of the defect in immune regulation, which is of course congenital with IEIs abut is often only encountered much later with ICIs. The magnitude of inhibition may also be important, since, as described above, there is a dose-response effect of observed with CTLA4 inhibition. Finally, it is plausible that microbiological challenge from infection may exacerbate the phenotype in humans, and that this could be less apparent in mice maintained under specific pathogen-free conditions.

AUTHOR CONTRIBUTIONS

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Primary Immune Deficiency: Patients' Preferences for Replacement Immunoglobulin Therapy

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Purpose: Immunoglobulin (Ig) replacement therapy is an important life-saving treatment modality for patients with primary antibody immune deficiency disorders (PAD). IVIG and SCIg are suitable alternatives to treat patients with PAD but vary in key ways. Existing evidence on patient preferences for Ig treatments given the complexities associated with IVIG and SCIg treatment is limited and fails to account for variations in preferences across patients. For this reason, we sought to evaluate PAD patient preferences for features of IVIG and SCIg across different patient characteristics.

Materials and Methods: 119 PAD patients completed a discrete-choice experiment (DCE) survey. The DCE asked respondents to make choices between carefully constructed treatment alternatives described in terms of generic treatment features. Choices from the DCE were analyzed to determine the relative influence of attribute changes on treatment preferences. We used subgroup analysis to evaluate systematic variations in preferences by patients' age, gender, time since diagnosis, and treatment experience.

Results: Patients were primarily concerned about the duration of treatment side effects, but preferences were heterogeneous. This was particularly true around administration features. Time since diagnosis was associated with an increase in patients' concerns with the number of needles required per infusion. Also, patients appear to prefer the kind of therapy they are currently using which could be the result of properly aligned patient preferences or evidence of patient adaptive behavior.

Conclusions: Heterogeneity in preferences for Ig replacement treatments suggests that a formal shared decision making process could have an important role in improving patient care.

Keywords: primary immune deficiency disorders, immunoglobulin replacement therapy, IVIg, SCIG, patient preferences, discrete-choice experiment

INTRODUCTION

Immunoglobulin (Ig) replacement therapy is an important life-saving treatment modality for patients with primary antibody immune deficiency disorders (PAD), especially those with antibody deficiency that account for approximately 50% of all types of primary immune deficiency disorders. The goal of treatment is to provide a broad spectrum of antibodies to prevent infections, inflammatory injury to vital organs like the lung, and chronic long-term complications.

Intramuscular gammaglobulin was first used in the early 1950s as replacement therapy until intravenous immunoglobulin (IVIG) was approved in 1981. This was a notable advancement since IVIG could essentially normalize the serum levels of IgG, and more productively protect patients from infection and even chronic lung disease. Clinical immunologists in Sweden took a different approach administering IVIG by the subcutaneous route. Gardulf et al. (1) and Ochs et al. (2) showed that the subcutaneous route for Ig replacement therapy, e.g. SCIg was safe, well tolerated, and effective in achieving adequate serum IgG levels. In a multicenter study of 165 patients with hypogammaglobulinemia receiving subcutaneous infusions (27,030 at home) a significant reduction in adverse systemic reactions was observed compared with intramuscular or intravenous administration. Although serious systemic reactions did not occur with SCIg, local tissue reactions did occur including swelling, soreness, redness, induration, itching, and bruising, but these were not serious and usually resolved with 48-72 hours. Thus, SCIg is a suitable alternative to IVIG and may present certain opportunities for optimizing at-home care for patients with PAD (3).

The SCIg products are 10%, 16.5% or 20% formulations; the 10% are products similar in composition to the IV product. Depending on the product, SCIg can be given biweekly, weekly or even more frequently as a subcutaneous push. The number of infusion sites varies from a single site to four sites depending on the product formulation (10% vs 20%), dosages, body weight of the patient and frequency (4).

A number of surveys have been published examining patients' health-related quality of life (HRQoL) (5) and treatment satisfaction with IVIG and SCIg replacement therapy in PAD patients. Several studies have shown enhancements in HRQoL with various treatment options, but it has been acknowledged that there is also "substantial treatment burden" and the burden can vary between the IV and SC routes, and site of care (5).

Multiple reports have shown that most patients choose home-based Ig replacement therapy and switch from receiving IVIG in a hospital to IVIG administered by a travel nurse, or SCIg self-administered in a home based setting (6–8). However, some evidence suggests that patients' perspectives could change with specific treatment experiences as Routes et al., (2016) found that about 88% of patients switched to IV administration at the hospital after 12 months of treatment (9).

Environmental and personal factors also can play a role in patients' preferences for PAD treatments. During the COVID-19 pandemic, some patients with PAD experienced high levels of anxiety and poor HRQoL when receiving hospital-based infusions. Others feared supply shortages while being treated at home (10, 11). The patient's job or lifestyle requirements also can affect their preferences, particularly if the patient must travel frequently (5). All of this highlights the importance of patients' perspectives in the selection of treatment options.

Particularly because IVIG and SCig are largely equivalent in terms of efficacy, the appropriateness of these options for a specific patient may be a matter of preference, or the relative importance of the features of each administration option. Recent studies have formally elicited stated preferences for treatments given the tradeoffs associated with IVIG and SCIg. This research typically differs from HRQoL evaluation tools in that it decomposes the relative importance of treatment factors to understand which aspects matter most to patients. Among PAD patients, this evidence has been rather limited (12, 13). Mohamed et al. (12), reported on patient and parent preferences for Ig replacement therapy attributes. Both parents and patients found that Ig administration in the home was preferable, with monthly frequency of the treatment using fewer needle sticks. A shorter duration of the treatment was also desirable. This work, however, did not assess the relationship between individual patient characteristics and treatment preferences.

While the available evidence on patient preferences suggests that at-home self-administration is generally preferred by patients, this perspective on treatment type is likely not universal. To date, little to no attention has been given to explaining what factors may be associated with different perspectives on treatments. Understanding the association between patient characteristics and treatment preferences can help patients and clinicians evaluate treatment options in a more efficient and meaningful way (14). Furthermore, understanding variations in patient preferences could help reduce treatment burden among patients who are not currently matched with their own preferred alternative.

This study evaluates stated preferences for attributes of IV and SC routes of administration of Ig replacement therapy for PAD patients with differing personal characteristics. We look to collect evidence on the association of patient characteristics with route of treatment preferences. Specifically, to evaluate whether the patients' age, years since diagnosis, gender and treatment experience made a difference in route of treatment choices.

MATERIALS AND METHODS

Adult patients with primary immune antibody deficiency who were members of the Immune Deficiency Foundation (IDF) or the Kantar Health Panel in the United States were invited to complete an online survey with a discrete-choice experiment (DCE). All respondents were required to have self-reported physician-diagnosis of PAD and to be able to provide consent.

The DCE was conducted following good-practice guidance (15). A DCE is a survey method that asks respondents to make choices between carefully constructed treatment alternatives where every treatment is described in terms of generic features

called attributes. In our case, these attributes included route of administration, number of needle sticks required for administration, treatment frequency, administration times, and side effects duration. Treatment choices differ from each other based on experimentally-controlled variations in their performance under each attribute (attribute levels).

To define the study attributes, we conducted a 90-minute focus group with a convenience sample of six adult patients with PAD in the Atlanta metropolitan area. From the focus group, we collected feedback on the aspects of treatments for PAD that patients most liked and disliked. We also collected information on patients' unmet needs, and treatment switching behavior and adherence. A comprehensive list of treatment-related aspects associated with the discussions during the focus group was defined based on participants' feedback. At the end of the focus group, participants completed an attribute-prioritization exercise using Case-1 Best-Worst Scaling (16) to determine the treatment attributes that would be included in the DCE. The resulting attributes and attribute levels are summarized in **Table 1**.

Based on the attributes and attribute levels selected, we developed a survey instrument with the input of preference researchers and clinical experts. The survey was pretested with a convenience sample of 5 adult patients with PAD, and 5 general-population respondents. Each individual interview was one-hour long and asked respondents to complete an online version of the survey instrument. During the pretest interviews, participants were asked to follow a think-aloud protocol. Respondents were asked to read the survey instrument out loud and were encouraged to articulate their thoughts related to survey information materials and questions. **Figure 1** presents the final choice question included in the survey.

The implementation of the DCE required the development of an experimental design with known statistical properties to populate the alternatives in the choice questions. We followed good-practice guidance on the development of the experimental design (17). Details on the experimental design can be found in **Appendix A**.

TABLE 1 | Attributes and attribute levels.

Attribute	Attribute Level
How the treatment is administered	Infusion under skin at home (no nurse) Infusion under skin at home (with nurse) Infusion under the skin at clinic (with nurse) Infusion into vein at home (with nurse) Infusion into vein at clinic (with nurse)
How many needle sticks	1 needle 2 needles 4 needles
How often you take the treatment	Once a month Twice a month 4 times a month
Administration time	1 hour 3 hours 6 hours
Time with headache and drowsiness	None 2 hours 10 hours 24 hours

Analysis

We first evaluated the validity of the DCE data based on commonly followed data quality checks, including response nonvariation in preferences (straight lining), attribute dominance, and attribute-comprehension questions (18). Additional information on these quality checks can be found in **Appendix A**. Respondents who were considered to be nonattentive based on these quality checks were excluded from the study sample. All mechanisms to address any observed quality issues were outlined prior to analysis.

After defining the final study sample, we followed good-practice guidance on the use of logit-based analysis to link patient responses to the tradeoffs required between the alternatives in the choice questions (19). Results from logit-based models produce preference weights in the form of log-odds (20). These weights reflect the average change in preferences for treatments with specific changes in attribute levels, all else equal. Additional details on the analysis of the preference data and the evaluation of variation in preferences can be found in **Appendix A**.

RESULTS

A total of 119 patients with PAD completed the survey instrument: 94 from the IDF and 25 from the Kantar Health Panel. **Table 2** presents the responses to the demographic and disease-experience questions included in the online survey instrument. The age distribution for respondents had a median of 51 years (range 18-77) Also, our sample was primarily female (87.4%). This is consistent with previous studies looking at quality of life outcomes among patients with PAD (8).

Time since diagnosis of PAD ranged from less than one year to 58 years, with a mean of 11 years since diagnosis and a median time of 8 years. About 77% of participants reported having experience with IVIG for the treatment of PAD. Meanwhile, 71.4% of respondents reported using SCig at some point to treat PAD. Nearly 49% (48.7%) of respondents reported having experience with both IVIG and SCig. Almost all participants (96.6%) reported that they currently receive infusions. Nearly 64% of them using SCig, while about a quarter of the respondents reported using IVIG. Most respondents (62.6%) self-administer their infusion at home.

No respondents were excluded from the final sample based on evidence of nonattention. We also found that no respondent made all treatment choices following the best level of a single attribute. However, 39 patients (32.8%) chose treatment based on the *number of needles* in at least 10 choice questions. Also, 4 patients (3.4%) chose treatment based on *frequency of treatment* in at least 10 choice questions. One patient (0.8%) chose treatment based on *administration time* in at least 10 choice questions, while 9 patients (7.6%) chose treatment based on *duration of side effects* in at least 10 choice questions. Finally, we

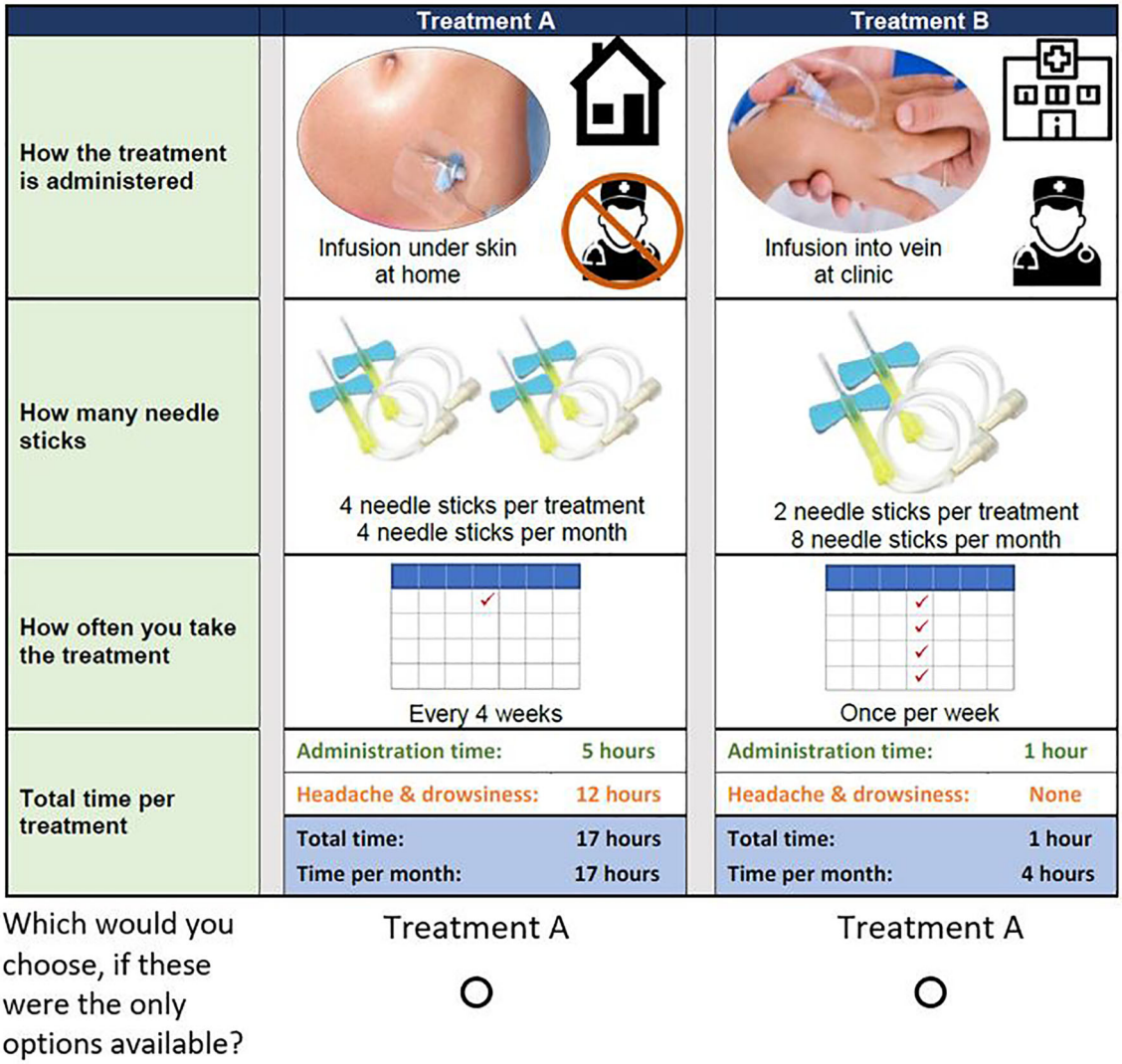

FIGURE 1 | Example DCE choice question. Example question answered by study participants. Respondents were asked to answer 14 of these questions, each with different combinations of levels for each treatment attribute.

found that 42.9% and 21.8% of patients incorrectly answered the first and second attribute-comprehension questions, respectively. About 13% of respondents (12.6%) answered both comprehension questions incorrectly. These were the questions meant to test the respondents' understanding of the DCE task layout. When respondents answered these questions incorrectly, we showed additional information to help them understand the concepts in the comprehension questions. We did not find that respondents who answered these questions incorrectly had different preferences from the rest of the sample (P>0.5).

We formally evaluated the functional form of the preference model with and without interaction terms between attributes but found that a main-effects specification had the best model fit. **Figure 2** plots the mean preference weights from the RPL model with the full sample and the 95% confidence interval for each attribute level. A table with the raw estimates from the RPL model, including the estimates for the random parameters can be found in **Appendix B**.

While the absolute value of the preference weights is not directly interpretable, higher preference weights indicate greater preference for a treatment with a specific attribute level. To facilitate the interpretation of the preference weights, we normalized all attributes so the most and least preferred attribute levels for duration of side effects had a value of 0 and -10, respectively (see **Figure 2**). All numeric attributes had the expected order of preferences (i.e., better clinical outcomes or less burdensome features were associated with higher preference weights). The differences in the level for route of administration (-1.15 to +1.15), setting (-1.44 to +1.44), and support from a nurse (-0.74 to +0.74) showed some of the smallest overall differences in

TABLE 2 | Demographic characteristics and respondent disease experience.

Statistic or Category	N = 119% (n)*
Age in years (as of Jan 1, 2019)	
Mean (SD)	48.5 (14)
Median	51
Minimum, Maximum	18, 77
Gender	
Female	87.4 (104)
Male	10.9 (13)
Other/Prefer Not to Answer	1.7 (2)
Race	
American Indian or Alaskan Native	0.0 ()
Asian	0.0 ()
African American	1.7 (2)
Native Hawaiian or Other Pacific Islander	0.0 ()
White	96.6 (115)
Other	1.7 (2)
Ethnicity	
Hispanic	1.7 (2)
Not Hispanic	98.3 (117)
Highest level of education completed	
Less than High School	2.5 (3)
High School Diploma/Equivalent	5.9 (7)
Some College	16.0 (19)
Associates Degree/Technical School	20.2 (24)
Bachelor's Degree	32.8 (39)
Graduate of Professional Degree	22.7 (27)
Marital status	
Single / never married	25.2 (30)
Married / living as married	58.0 (69)
Divorced or separated	16.0 (19)
Widowed / surviving partner	0.8 (1)
Other	0.0 ()
Do you have children younger than age 18 or other dependents who live with you at home?	
Yes	17.6 (21)
No	82.4 (98)
Employment status	
Employed/Student	46.2 (55)
Retired	16.0 (19)
Disabled	29.4 (35)
Not Currently Employed	8.4 (10)
Time since diagnosis in years (as of Jan 1, 2019)	
Mean (SD)	11.0 (10.8)
Median	8
Minimum, Maximum	<1, 58
Methods ever used to manage PAD symptoms	
Take prescription pills or tablets	74.8 (89)
Received extra vaccines	31.9 (38)
IVIG (Intravenous immunoglobulin infusion) treatment	76.5 (91)
SCIg (Subcutaneous immunoglobulin infusion) treatments	71.4 (85)
Bone marrow transplant	0.0 ()
Changed my lifestyle or exercise routines	57.1 (68)
Acupuncture, chiropractic adjustments, or dietary supplements	48.7 (58)
None of the above	0.0 ()
Currently receiving infusions	96.6 (115)
Which option is closest to the way you receive infusions?	(n=115)
Infusion into the fatty layer under the skin	63.5 (73)
Infusion into a vein in my arm or hand	24.3 (28)
Another kind of infusion (for example, through a port or PICC line)	12.2 (14)
Where are your infusions received?	(n=115)
A nurse comes to my home to administers the infusion	13.9 (16)
I administer the infusion at home without a nurse	62.6 (72)
I go to a clinic where a nurse administers my infusion	22.6 (26)
Other	0.9 (1)
Side effects from last treatment	(n=115)
Headache	46.1 (53)
Tiredness / fatigue	73.0 (84)
Nausea	18.3 (21)
Rash or skin reaction	23.5 (27)
Itchiness	22.6 (26)
Other	15.7 (18)
No side effects	16.5 (19)

Unless otherwise noted. †Percentages do not add up to 100% across response categories because respondents were allowed to select multiple answers.

preference weights. On average, self-administration of SC therapies at home was most preferred by respondents.

Differences in preference weights between attribute levels are considered the importance of that attribute change. When we consider the most and least preferred levels for an attribute, this difference represents the attributes maximum importance relative to the other features in the study. This is also commonly called overall attribute importance (21). We can normalize that overall attribute importance to evaluate how much each attribute mattered in the DCE tasks presented to respondents. **Figure 3** presents these overall importance values using profile-based normalization (22).

The most important attribute given the range of levels covered in the study was duration of side effects (31.88%), followed by frequency of treatment administration (25.47%), and number of needles required for administration (12.36%). The least important attributes were whether the treatment was administered by a nurse (4.71%), route of administration (7.35%) and treatment setting (9.19%).

Normalized attribute importance does not just indicate the ranking of attributes, but can be used to determine the relative intensity of attribute importance. For example, duration of side effects (31.88%) was approximately 3 times more important than infusion time (9.04%) and treatment setting (9.19%). Meanwhile, frequency of administration (25.47%) was about as important as the three attributes associated with self-administration combined (setting (9.19%), nurse support (4.71%), and route of administration (7.35%) (**Figure 3**).

We evaluated preference heterogeneity based on four patient characteristics: age, years since diagnosis, gender, and previous experience with IVIG and/or SCIg. We failed to reject a hypothesis of equal preferences based on age (respondents above and below the age of 65)(*P-value*=0.83), and gender (female versus males)(*P-value*=0.91). This means that there was not enough information in our data to say that older and younger respondents had different preferences. The same was true of differences between men and women who completed the DCE.

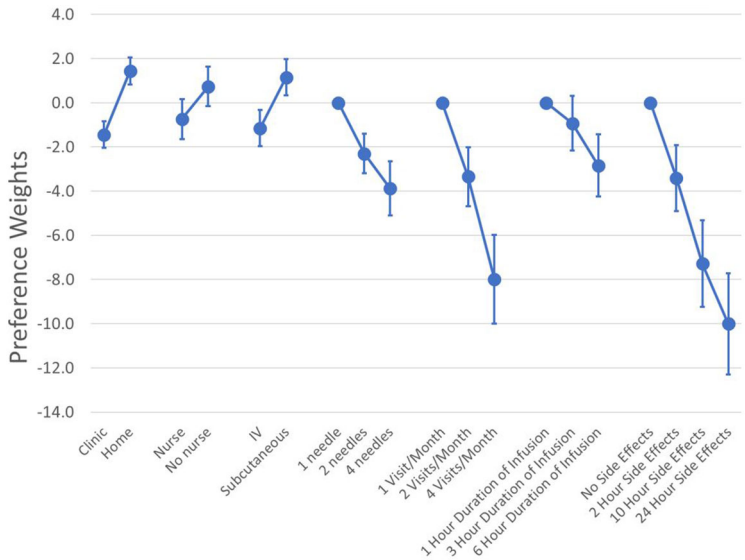

FIGURE 2 | Mean preference weights (N = 119). Log-odds preference weights for all respondents. The absolute value of the weights has no direct meaning. What matters is the relative size of the vertical differences between preferences weights. This is because that vertical distance is correlated with changes in the probability of choice given the attribute change. For example, increasing the duration of infusions from 1 hour to 6 hours reduced the preference weights from 0 to -2.8. Similarly, an increase in the time with side effects from no side effects to 10 hours of side effects decreased the preference weights from 0 to -7.3. This means that the 10-hour increase in the duration of side effects was about 2.6 times (2.6 = -7.3/-2.8) as important as 5-hour increase in the administration time.

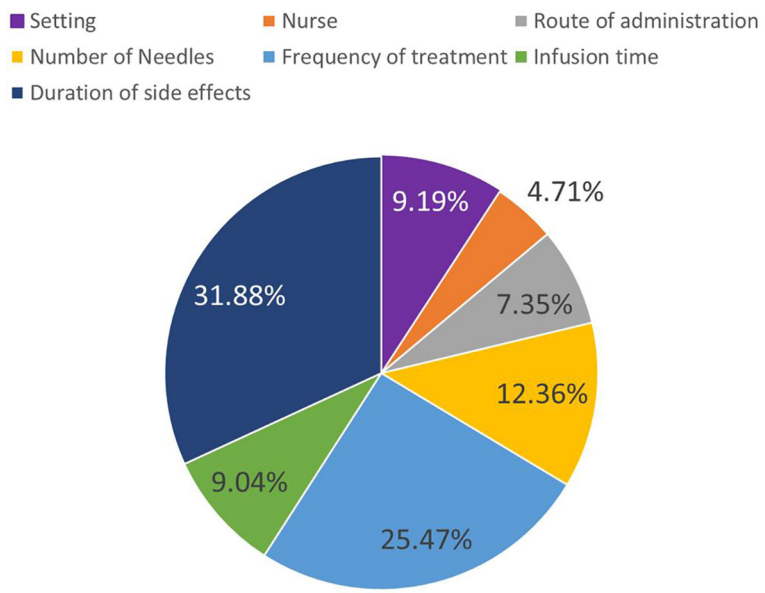

FIGURE 3 | Overall attribute importance. Overall attribute importance weights depict the most influence an attribute change had on treatment choices. This is based on the biggest preference-weight difference within each attribute.

We found that patients with different number of years since diagnosis had different preferences on average. Changes in attribute levels had different impacts on treatment choice across patients who were diagnosed at least 8 years ago (median time since diagnosis in our sample), and those diagnosed more recently (**Figure 4**). Differences in preferences among these subgroups are represented by variations in the vertical distance between point estimates within attributes. Similarly, we found that preferences varied across patients with different treatment experiences (*P-value*<0.001 for IVIG

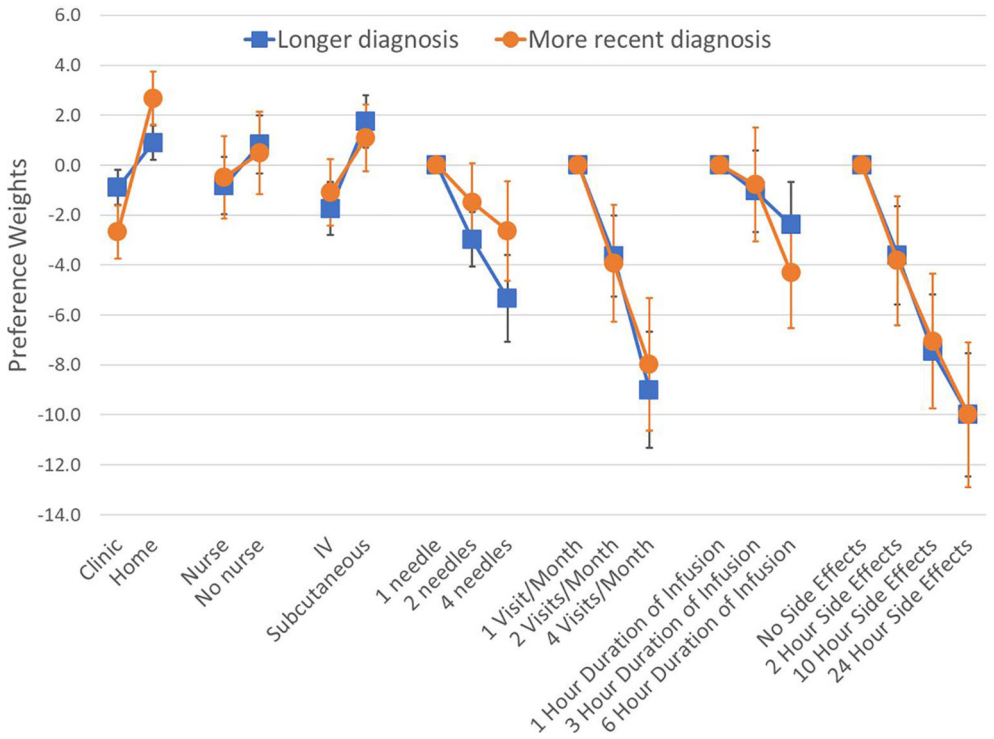

FIGURE 4 | Preference weights by time since diagnosis. Log-odds preference weights for respondents with longer (>8yrs ago) versus more recent diagnosis (<8yrs ago). Lines around each estimate indicate the 95% confidence interval. Results were normalized by overlapping the preference weights for duration of side effects to allow direct comparison between plots. Statistically-significant differences between the groups were found for the administration setting.

experience, and *P-value*=0.042 for experience with SCIg) (**Figure 5**). As before, all preference weights in each subgroup were normalized so the most and least preferred attribute levels for duration of side effects had a value of 0 and -10, respectively.

We found that across subgroups, respondents generally still preferred to be treated at home. However, respondents who were diagnosed less than 8 years ago were almost three times more concerned about treatment setting than those with longer diagnosis. Regarding treatment experience, results show that respondents who only have IVIG experience prefer using IV therapies and having a nurse administer the treatment. Those who only had experience with SCIg were less concerned about needles and preferred self-administration. Finally, respondents who reported having experience with both IVIG and SCIg appear to be indifferent between the two routes of administration. These respondents also were concerned about the number of needle sticks, side effect duration and number of visits, but preferred self-administration at home. Finally, the patient group with no prior therapy had preferences for SCIg, no nurse and at-home for treatment.

DISCUSSION

Our study looked to quantify the preferences of patients with PAD based on the factors that most influence their views about treatments. We set out to accomplish this by developing and implementing a DCE. Our results suggest that these patients have well-defined preferences for the attributes we considered in the study.

On average, the patients in our study were primarily concerned about the duration of treatment side effects. Among the process factors considered (excluding health outcomes like side effects), frequency of administration was the most important attribute. We also found that the average respondent seemed to prefer self-administration at home without a nurse. These results are consistent with previously published work on preferences and HRQoL for immunoglobulin therapies (1, 5, 8, 12, 13, 23). However, contrary to Mohamed et al. (12), we did not find significant interaction effects between frequency of administration and duration of administration, duration of side effects, and number or needles. This means respondents did not seem to expect varying levels of disutility from any of these attributes as frequency of treatments increased.

Although generally respondents showed preference for SCIG, the specific dosing given to patients seems to be relevant in an ultimate decision between treatment types. We found that nearly a third of patients chose treatments based on the number of needle sticks in at least 10 of 14 questions. This suggests strong aversion to needles by some respondents. Also, given the levels in our experiment, treatment frequency was about as important as setting, support from a nurse and route of administration.

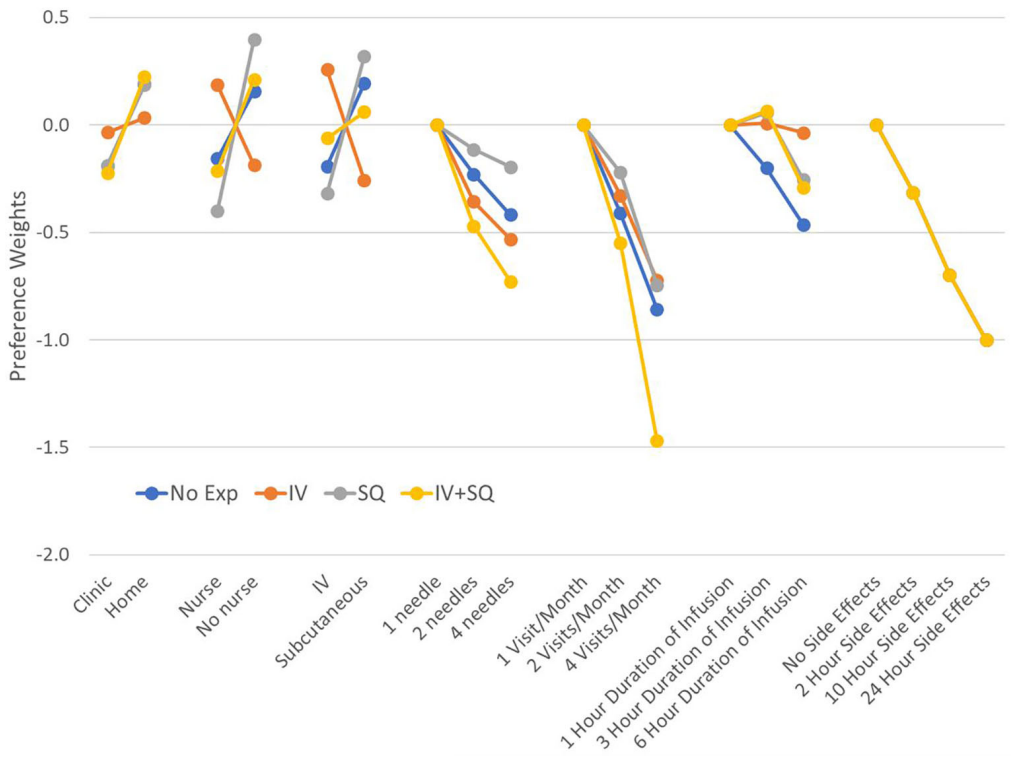

FIGURE 5 | Preference weights by treatment experience. Log-odds preference weights for respondents who reported only using IV therapies (IV), those who reported only experience with (subcutaneous injections), and those who reported experience with both administration options. Results were normalized by overlapping the preference weights for duration of side effects to allow direct comparison between plots. Confidence intervals are not shown to facilitate reading the figure. Estimates and 95% confidence intervals for each subgroup are included in **Appendix B** No exp, No experience with any therapy; IV, Only experience with IVIG; SQ, Only experience with subcutaneous injections; IV+SQ, Experience with both IVIG and subcutaneous injections.

This implies that, on average, patients would be more concerned about the frequency of treatment than the process features associated with IVIG and SCIG. In other words, a less frequent IVIG could look more attractive than a more frequent SCIG.

While respondents in our sample appeared to have well-defined preferences, those preferences were not homogeneous across patients. Both time since diagnosis and treatment experience were correlated with variations in preference weights. Increased time since diagnosis was associated with greater concern with the number of needles required, while experience with a specific treatment type was associated with greater preference for that treatment (IVIG vs. SCIG). The latter may indicate one of two things: 1) patients are already receiving the treatments they want, or 2) they develop affinity for the attributes of the treatments they receive. Either way, our results suggest that at least some patients with PAD may be averse to treatment switching.

The aversion to treatment switching could imply that a formal treatment shared-decision process could facilitate treatment-initiation or treatment-switching discussions and help physicians convey the benefits of different treatment types. Similar efforts have previously shown to have an impact in treatment acceptance and quality of life among patients with common variable immune deficiency. (24) With this in mind, preference-based tools in support of shared decision making could also help improve treatment adherence and outcomes.

It is worth noting some key limitations of our study. The survey elicits preferences between hypothetical treatment options. The recorded choices do not carry the same consequences as real-world treatment decisions. While the choices elicited here might be different from those made in a clinical context, the study team followed best practices in survey research to make the questions consequential and to induce preference-revealing answers (25). Another important limitation is that the relative importance of the attributes elicited through the DCE are conditional on the attributes and attribute levels included in the study. That said, these attributes and levels were defined with direct patient input and in consultation with clinical experts. Finally, while the characteristics of survey respondents were largely consistent with samples from previous studies conducted in this population (9, 24), our sampling framework does not guarantee that our preference estimates are representative of the broader PAD patient population. Despite potential issues with the representativeness of the study sample, the identified variations in preferences suggest there are systematic differences in the acceptability of the tradeoffs implied by specific treatment options.

CONCLUSIONS

The majority of patients with PAD in our study wanted to be treated at home, but we found that setting or route of administration represent a relatively small part of patients' preferences for treatments, so treatment dosing could overcome the benefits of treatment route of administration. We also found that patient preferences for treatments were not homogeneous across patients. Treatment experience can be associated with preferences for IV administration with a nurse. These heterogeneous views on the relative importance of aspects of treatments, suggests that a formal shared decision making process could have an important role in improving patient care, particularly if patients indeed are adapting to therapies that may result in unnecessary treatment burden. Such a proposal is not new (9, 26) and instruments like the one developed for this study could be adjusted to help document patients' views in a clinical setting. The information collected through such a preference-elicitation tool could support open discussions around the tradeoffs that patients are willing to accept between treatment aspects, and potentially help minimize HRQoL impacts of treatments by adequately matching patients' preferences and treatment options.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Duke University Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JG led the study design, survey development, data analysis and the drafting the manuscript. MB contributed to the study design, survey development, data analysis and drafting of the manuscript. AF contributed to the study design, survey development, data analysis, and provided critical input to the development of the manuscript. CR contributed to the study design, survey development, data analysis and provided critical input to the development of the manuscript. All authors contributed to the article and approved the 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.2022.827305/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antigen-Specific CD4$^+$ T-Cell Activation in Primary Antibody Deficiency After BNT162b2 mRNA COVID-19 Vaccination

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Previous studies on immune responses following COVID-19 vaccination in patients with common variable immunodeficiency (CVID) were inconclusive with respect to the ability of the patients to produce vaccine-specific IgG antibodies, while patients with milder forms of primary antibody deficiency such as immunoglobulin isotype deficiency or selective antibody deficiency have not been studied at all. In this study we examined antigen-specific activation of CXCR5-positive and CXCR5-negative CD4$^+$ memory cells and also isotype-specific and functional antibody responses in patients with CVID as compared to other milder forms of primary antibody deficiency and healthy controls six weeks after the second dose of BNT162b2 vaccine against SARS-CoV-2. Expression of the activation markers CD25 and CD134 was examined by multi-color flow cytometry on CD4$^+$ T cell subsets stimulated with SARS-CoV-2 spike peptides, while in parallel IgG and IgA antibodies and surrogate virus neutralization antibodies against SARS-CoV-2 spike protein were measured by ELISA. The results show that in CVID and patients with other milder forms of antibody deficiency normal IgG responses (titers of spike protein-specific IgG three times the detection limit or more) were associated with intact vaccine-specific activation of CXCR5-negative CD4$^+$ memory T cells, despite defective activation of circulating T follicular helper cells. In contrast, CVID IgG nonresponders showed defective vaccine-specific and superantigen-induced activation of both CD4$^+$T cell subsets. In conclusion, impaired TCR-mediated activation of CXCR5-negative CD4$^+$ memory T cells following stimulation with vaccine antigen or superantigen identifies patients with primary antibody deficiency and impaired IgG responses after BNT162b2 vaccination.

Keywords: circulating follicular T helper cells, CXCR5-negative CD4$^+$ memory T cells, common variable immunodeficiency, primary immunoglobulin isotype deficiency, activation induced marker assay, surrogate virus neutralization assay

INTRODUCTION

COVID-19 (Coronavirus Disease-2019) is caused by infection with SARS-CoV-2, a novel coronavirus discovered at the end of 2019 (1). Interaction between angiotensin converting enzyme 2 (ACE2) highly expressed on human airway epithelial cells and the receptor binding domain of the viral spike protein mediates entry of SARS-CoV-2 into the cell, thereby establishing infection of the host (2). SARS-CoV-2-infected individuals may develop potentially life-threatening pneumonia and respiratory failure in the course of a severe acute respiratory syndrome (SARS) associated with high mortality (3).

Defects of innate and adaptive immunity such as impaired type I interferon response (4), loss of function variants of the X-chromosomal TLR7 gene (5) or predominantly antibody deficiency (PAD) can be responsible for severe COVID-19 with high hospitalization and infection fatality rates (6, 7). Among the PAD group patients with common variable immunodeficiency (CVID) complicated by inflammatory, autoimmune or respiratory comorbidities were most vulnerable to develop severe COVID-19 (6–8). CVID is the most common clinically severe form of primary antibody deficiency, characterized by a severe impairment to produce pathogen-specific IgG antibodies (9). Other forms of PAD (oPAD) show a persistent and marked decrease of at least one of the serum immunoglobulins and/or IgG-subclasses and/or a specific antibody deficiency to polysaccharide antigens but have an intact ability to produce IgG antibodies after vaccination with T-dependent protein antigens (10). Although clinical presentation of these patients is often mild as compared to CVID, severe disease can still develop (11) and these patients can also present with severe COVID-19 (8).

The most effective protection against infection with SARS-CoV-2 is achieved through induction of antibody and T cell responses following vaccination against SARS-CoV-2 spike protein, e.g., with BNT162b2, a new COVID-19 mRNA vaccine (12, 13). As in other viral infections vaccine-induced neutralizing IgG antibody responses are generally considered to be a surrogate marker for immune protection (14, 15). CVID patients might still be susceptible to infection after vaccination as impaired IgG antibody and B cell memory responses have been described following immunization with conventional vaccines, e.g., against seasonal influenza (16, 17) and also with BNT162b2 in one study (18) but not in another (19). The mechanism whereby in a subgroup of CVID patients BNT162b2 vaccination induces IgG antibody formation (18, 19) remains to be studied. In addition, immune responses to BNT162b2 in patients with immunoglobulin isotype deficiency and/or selective antibody deficiency, known to be capable of producing IgG antibodies to conventional vaccines, have not been studied in detail yet.

The generation of B cell memory is critical for the efficacy of a vaccine. $CD4^+$ T follicular helper (Tfh) cells promote long-lived humoral immunity after vaccination by providing help to B cells in germinal center reaction in follicles of secondary lymphoid organs (20, 21). Circulating T follicular helper cells (cTfh) contained within the $CXCR5^+$ memory $CD4^+$ T cell compartment in peripheral blood reflect the Tfh present in germinal center follicles of secondary lymphoid organs (20). In convalescent individuals, SARS-CoV-2-specific cTfh responses correlate with antibody neutralization found within two months following symptoms onset (22), and cTfh responses also participate in IgG antibody production to SARS-CoV-2 spike protein contained in the currently available vaccines (23). In CVID patients divergent results have been reported regarding vaccine-specific T cell responses as identified by IFN-gamma production. While intact T cell responses were found following influenza (24) or BNT162b2 vaccination (19), vaccine-specific T cells did not increase following BNT162b2 immunization in another more recent study (18). CTfh abnormalities have been described in CVID patients (25), but cTfh responses following mRNA vaccination against COVID-19 have not been studied in patients with PAD. In the present study we investigated antigen-specific $CD4^+$ T memory subset response using an activation-induced marker assay (26), and formation of IgG and IgA antibodies and also surrogate virus neutralization antibodies against SARS-CoV-2 spike protein by ELISA in patients with PAD vaccinated with two doses of BNT162b2 mRNA vaccine. Our findings indicate that the majority of PAD patients produce normal levels of functional IgG antibody responses following BNT162b2 vaccination and should thus be protected from COVID-19. In addition, abnormalities in vaccine-specific $CD4^+$ T cell responses characterize PAD patients with defective IgG responses.

PATIENTS AND CONTROLS

A total of 31 adult patients diagnosed with CVID according to the ESID registry working definitions for the clinical diagnosis of inborn errors of immunity (10) were enrolled in the study (median age in years [IQR] (range): 45 [37–57] (19–85); m/f 12/19) (**Table 1**). All CVID patients received regular SCIG or IVIG substitution therapy and had never experienced a previous SARS-CoV-2 infection as nasopharyngeal swabs were repeatedly negative by PCR testing; the patients lived in social isolation and experienced no clinical symptoms indicative of viral infection. Serum and peripheral blood mononuclear cells were collected 40 days (median, IQR: 34–57) following the second dose of the Pfizer-BioNTech COVID-19 vaccine BNT162b2 (Comirnaty) to determine vaccination responses as part of the routine medical attendance. Monogenetic variants known to be associated with CVID were present in three patients: an IKZF1 mutation in two IgG-responders and an NFKB1 mutation in one IgG-nonresponder; in all other CVID patients the presence of gene mutations associated with other primary immunodeficiency disorders, and also any known monogenetic cause of CVID phenotype was excluded by targeted gene sequence analysis (Illumina technology performed on a MiSeq NGS). Eleven CVID patients without any exposure to SARS-CoV-2 were tested before they received COVID-19 vaccination. For comparison 39 patients with other, milder forms of predominantly antibody deficiency (oPAD) were included in the study (median age in years [IQR] (range): 52 [38–71] (18–90); m/f 13/26), 28 with regular immunoglobulin substitution therapy (fifteen of these had selective

Abbreviations: CVID, common variable immunodeficiency; HC, healthy controls; oPAD, milder forms of primary antibody deficiency other than CVID, e.g. immunoglobulin isotype deficiency or selective antibody deficiency; SEB, Staphylococcal enterotoxin B; cTfh, circulating T follicular helper cell; IQR, interquartile range.

TABLE 1 | Characterization of CVID patients with or without IgG-antibody production following second BNT162b2 mRNA COVID-19 vaccination.

	CVID responders (n=15)			CVID nonresponders (n=16)			Normal range
sex (m/f)	4/11			8/8			
	median	IQR		median	IQR		
age at time of diagnosis (yr)	34	26.5	46.4	32	26.6	47.3	
age at time of second BNT162b2 vaccination (yr)	49	36.5	58.5	42	36.5	55	
days between second vaccination and testing of immune response	40	35.5	50.5	40	32.8	61	
serum immunoglobulins (mg/dl)							
IgG	363	221.8	558	121	76.5	218	790-1700
IgA	<6	<6	34.5	<6	<6	8	76-450
IgM	37	18.3	60.8	16	<6	63	90-350
serum antibody levels							
23-valent PnP-IgG (reciprocal titer)	<20	<20	<20	<20	<20	<20	428-10785
23-valent PnP-IgM (reciprocal titer)	25	<20	38.5	<20	<20	<20	164-11943
Tet-IgG (IU/ml)	0.13	0.12	0.35	0.09	0.04	0.24	1.67-12.14
Di-IgG (IU/ml)	0.04	0.01	0.19	0.01	0.005	0.02	0.42-7.22
Hib-IgG (ug/ml)	0.15	0.11	0.29	0.11	0.06	0.45	>1
Lymphocyte subpopulations							
CD19 % of lymphocytes	13	10.64	15	7	5.07	13.3	7-23
CD19 abs. number/ul	164	142.75	271.5	88	53.5	250	71-549
CD4 % of lymphocytes	39	33.8	43.25	40	32.5	45	31-66
CD4 abs. number/ul	457	377	579	640	509.75	708.5	386-2022
CD8 % of lymphocytes	36	26.6	36.7	38	31.27	52	21-43
CD8 abs number/ul	342	253	564	547	436	1192	297-1011
B cell subpopulations (% of CD19-positive lymphocytes)							
IgD+CD27-	73	65.37	85.38	86	77.9	91.3	45.84-80.36
IgD+CD27+	23	10.46	29.42	8	4.29	16.9	6.81-30.53
IgD-CD27+	3	2.01	5.05	2	0.76	2.28	7.81-27.45
Lymphocyte proliferation (dpm)							
PHA	98790	62713	162669	92454	582373	131827	>20000

antibody deficiency against polysaccharide antigens (SPAD), ten with concomitant IgG subclass deficiency; 13 had IgG subclass deficiency without SPAD), eleven without immunoglobulin replacement (one had selective IgG2-antibody deficiency, six had IgG subclass deficiency, two with concomitant IgA deficiency, four had IgM deficiency). A median of 35 days (IQR 28–59) following the second dose of Pfizer-BioNTech COVID-19 vaccine their immune responses were tested. Eleven oPAD patients without any exposure to SARS-CoV-2 were tested before vaccination. Twenty healthy adults [median age in years (IQR) (range): 62 (47–67) (26–73); m/f 5/15] served as a control group and were tested 39 days (median, IQR: 24–54) following the second dose of Pfizer-BioNTech COVID-19 vaccine; sixteen healthy adults who refused to get a COVID-19 vaccine and were repeatedly tested negative for SARS-CoV-2 infection wanted to know whether they were protected from COVID-19 and served as a SARS-CoV-2-negative healthy control group.

MATERIALS AND METHODS

Measurement of Antibody Responses After BNT162b2 Vaccination

Antibody response was evaluated by testing IgG- and IgA-antibodies against the recombinant S1 domain of SARS-CoV-2 spike (S) protein (containing the receptor-binding domain) with commercially available quantitative [anti-SARS-CoV-2-Quantivac-ELISA (IgG), Euroimmun Medizinische Labordiagnostika AG, 23560 Lübeck, Germany] and semiquantitative [anti-SARS-CoV-2-ELISA (IgA), Euroimmun Medizinische Labordiagnostika AG] ELISA kits used according to the manufacturer's instructions. Results for IgG antibodies are expressed as relative units (RE)/ml, values ≥11 RE/ml are considered positive. These relative units can be converted to binding antibody units (BAU) according to the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (27) by multiplication with a factor of 3.2. Semiquantitative measurements of IgA antibodies are expressed as the ratio between the extinction of patient samples and the extinction of a calibrator provided with the kit; values of ≥1.1 are considered as positive IgA antibodies. Surrogate virus neutralizing (sVNT) antibodies were assessed using the cPass SARS-CoV-2 Neutralization Antibody Detection Kit (Nanjing GenScript Biotech Co., Ltd., 211100 Nanjing City, P.R. China) according to the manufacturer's instructions. This blocking ELISA can detect antibodies that inhibit the interaction between recombinant SARS-CoV-2 receptor-binding domain and angiotensin-converting enzyme 2 in an isotype-independent manner (28, 29). Results are expressed as percent inhibition calculated according to the following formula: % inhibition = [1 – (OD value of sample/OD value of negative control)] × 100; results with ≥30% inhibition were considered positive indicating the presence of SARS-CoV-2 neutralizing antibody.

Examination of Antigen-Specific Circulating Follicular T-Helper and CXCR5-Negative CD4+ Memory T-Cells

An activation-induced marker assay was used to detect T cells specific for SARS-CoV2 spike protein (26). Human peripheral

blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation (Lymphoprep; Invitrogen, Lofer, Austria) and cultured in RPMI 1640 medium (GibcoBRL, Invitrogen) containing 2 mM/ml L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) and supplemented with 10% heat inactivated fetal calf serum (Gibco, Paisley, UK) as previously described (30) at 37°C in the presence of 5% CO2 and 95% humidified air in 24-well tissue culture plates (TC-Platte 24 Well, Standard, F; Sarstedt AG & Co. KG, Nürnbrecht, Germany) in a concentration of 1×10^6 cells/ml and well for two days in the presence of overlapping peptides of immunogenic regions of SARS-CoV2 spike protein (Peptivator SARS-CoV-2 Prot_S, Order.No.: 130-126-701, Miltenyi Biotec) (1 µg of peptides/ml) or the bacterial superantigen staphylococcus enterotoxin B (SEB) (31) in a final concentration of 1 µg/ml. Control cells were incubated in parallel in culture medium only (unstimulated cells). $CD4^+$ T-cell subpopulations were then characterized by flow cytometry (32) using commercially available monoclonal antibodies against CD3, CD4, CD45RA, CXCR5 and CXCR3, while upregulation of CD25 and CD134 (OX40) was used to identify activation of $CD3^+/4^+/45RA^-/CXCR5^-$ (CXCR5-negative memory T-helper cells, Tmem) and $CD3^+/4^+/45RA^-/CXCR5^+$ (circulating follicular T-helper cells, cTfh) cells. Cells were acquired with a FACSVerse (Becton Dickinson; USA) according to the manufacturers recommendations and analyzed using FACSuite software (Becton Dickinson; USA). A lymphocyte and singlet gate was applied, thereby excluding dead cells and cell debris, and at least 100,000 events within this "lymphogate" that were CD3-positive were acquired. The CD3/CD4/CD8/CD45RA/CXCR5 gating strategy used in flow cytometric analysis is depicted in **Figure 1**, panel A. Results are expressed as percent activated (CD134 and CD25 double positive) cells relative to the respective $CD4^+$ T cell subpopulation (see **Figure 1** for cell gating strategies and activation marker expression). Alternatively, induction of CD69-expression on activated cTfh and Tmem following stimulation of PBMC with SARS-CoV-2 spike peptides was examined by flow cytometry. Intracellular TNF-alpha expression was examined by flow cytometry after PBMC were stimulated with overlapping peptides of immunogenic regions of SARS-CoV2 spike proteins for two days before brefeldin A was added to block the Golgi-Apparatus and allow for accumulation and detection of intracellular cytokines using a standard protocol. Unstimulated PBMCs were incubated in parallel in medium alone. For measurement of SARS-CoV-2 spike peptide-specific lymphocyte proliferation PBMCs were stimulated for seven days using SARS-CoV2 Spike peptides (1 µg of peptides/ml) before 3H-thymidine was added for 16 h and 3H-thymidine incorporation was assessed as previously described (30). Results are expressed as netto dpm of 3H-thymindine incorporation, calculated by subtracting dpm of unstimulated cell from dpm of stimulated cells.

Statistical Analysis

Comparison of three or more study groups was performed by calculating the Kruskal–Wallis one-way analysis of variance using Graphpad Prism 6.0.7 software (GraphPad Software, San Diego, CA 92108). Statistical differences between two groups were then confirmed by using the non-parametric two-tailed Mann–Whitney U-test. Values of $p < 0.05$ were considered as statistically significant.

Data Sharing

Deidentified individual participant data that underlie the reported results will be made available 3 months after publication for a period of 5 years after the publication. Please send all inquiries to hermann.wolf@itk.at.

RESULTS

Antibody Responses After BNT162b2 Vaccination Are Impaired in a Subgroup of Patients With CVID

Healthy controls, oPAD- and CVID-patients had significantly higher SARS-CoV-2 S-protein-specific IgG antibody levels after BNT162b2 vaccination as compared to individuals tested before vaccination (**Figure 2A**). IgG responses in CVID patients as a group however were significantly lower as compared to both healthy controls and oPAD patients (**Figure 2A**); sixteen (51.5%) of 31 CVID patients showed anti-S-IgG antibodies below the detection limit or very low to borderline (below 33 RE/ml, which is three-times the detection limit of 11 RE/ml), while 15 (48.4%) CVID patients produced levels of IgG antibodies comparable to healthy controls (HC) (**Figure 2A**). The IgG antibody level used to separate responders from non-responders was defined following the arbitrary but commonly used rule to define robust, durable and generally more significant IgG antibody levels as those above three-times the detection limit. Among 16 CVID non responders, four patients had IgG antibodies that were low to borderline detectable with levels between 12.5 and 22.5 RE/ml. Only one of these four showed detectable antibodies of 49.9% inhibition in the surrogate neutralization assay. oPAD patients displayed IgG antibody levels after BNT162b2 vaccination that were comparable to healthy controls (**Figure 2A**), and 30/39 oPAD patients (77%) were above the 5% quantile of the healthy control group.

In CVID patients, serum IgA antibodies against spike protein were clearly lower than HC and oPAD, with levels that were comparable to unvaccinated individuals (**Figure 2B**); only four of 31 CVID patients (12.9%) produced detectable anti-S IgA antibodies after two vaccinations (**Figure 2B**). Anti-spike IgA levels were significantly higher in oPAD patients or healthy controls after vaccination as compared to values obtained in unvaccinated individuals, but there was a trend towards lower levels in oPAD patients as compared to healthy controls (IgA anti-spike, median ratio: HC 9.10; oPAD 3.25), and seven oPAD patients failed to produce any IgA antibodies, including two patients with IgA deficiency (**Figure 2B**).

In all three study groups sVNT antibodies were significantly higher after two doses of mRNA COVID-19 vaccination as compared to values detected in vaccine-naïve individuals (**Figure 2C**). Sera from CVID patients that contained significant levels of anti-spike IgG antibodies (>33 RE/ml) also showed

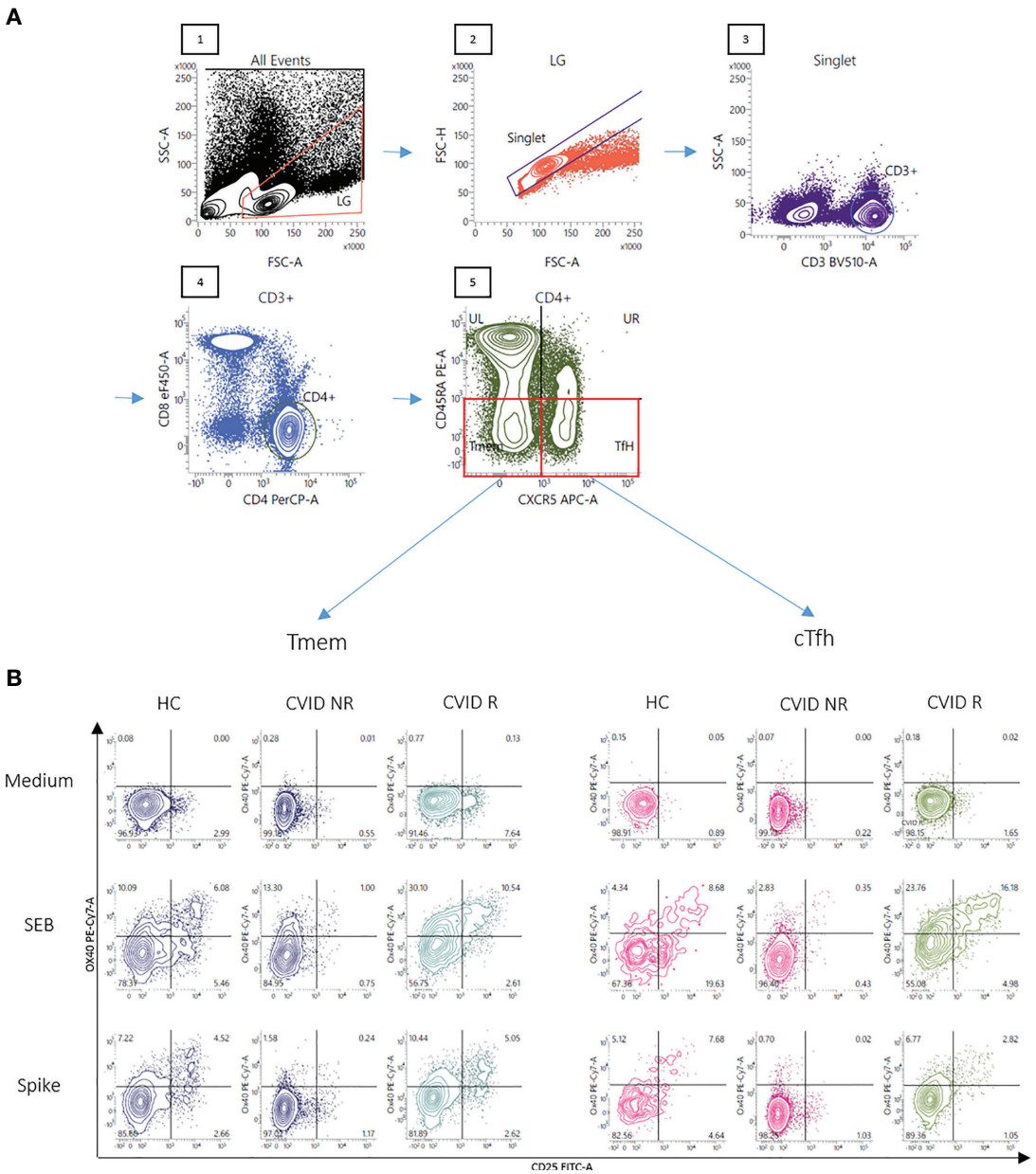

FIGURE 1 | Gating strategy and representative FACS plots of CD4+ T memory cells responsive to SARS-CoV-2 spike peptides. Panel **(A)** A lymphocyte gate was applied to a forward - sideward scatter plot of all events (1), followed by doublet exclusion using forward scatter area vs height for cells within the lymphocyte gate (LG) (2). $CD3^+$cells were then selected out of singlets (3) and $CD3^+$ T-cells were examined for CD4 staining (4). Finally $CD3^+CD4^+$ $CD45RA^-$ T-memory cells were divided into $CXCR5^-$ (Tmem) and $CXCR5^+$ (cTfh) cells (5). $OX40(CD134)^+CD25^+$ cells were finally defined as activated T-cells. Panel **(B)** Activation of Tmem and cTfh cells after stimulation of the cells with SEB or overlapping peptides of SARS-CoV-2 spike protein is shown in a representative healthy control (HC), a CVID patient IgG-responsive to BNT162b2 vaccination (CVID R, SARS-CoV-2 IgG antibody level following the second vaccination more than 33 RE/ml) and a CVID patient IgG-non-responsive following mRNA vaccination (CVID NR, SARS-CoV-2 IgG below 33 RE/ml following the second vaccination). Unstimulated control cells were incubated in medium alone (Medium). The percentages of $OX40(CD134)^+$ and $CD25^+$ double-positive activated CD4 T cell subsets are shown in the upper right panel of the respective FACS plots.

significant neutralizing capacity (>30% inhibition) in the sVNT ELISA. In addition, one CVID patient with borderline to low anti-spike IgG antibodies (22.5 RE/ml) showed positive sVNT antibodies (49.9% inhibition), indicating that even moderately low titers of IgG antibodies can have neutralizing activity, at least in the sVNT assay, and one CVID-patient that produced IgA-antibodies (IgA ratio 10.6) but no IgG-antibodies showed inhibitory antibodies in the sVNT assay (76.7% inhibition), indicating that anti-spike IgA antibodies might be responsible for virus neutralization in this patient. In terms of antibody functionality

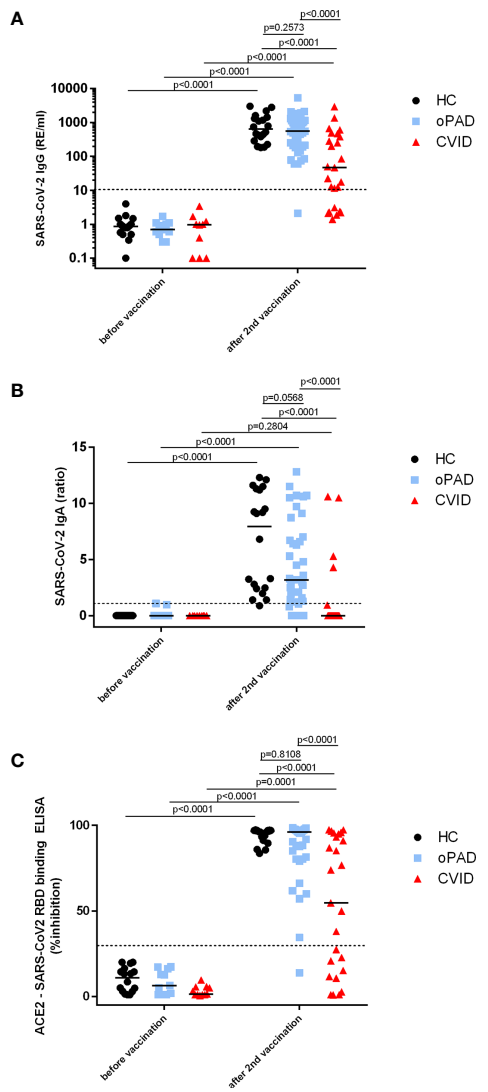

FIGURE 2 | Antibody response following a second BNT162b2 mRNA vaccination in patients with CVID and oPAD. Serum IgG **(A)** and IgA **(B)** antibodies against spike protein of SARS-CoV-2 were examined by ELISA in patients with CVID, patients with milder forms of primary antibody deficiency (other predominantly antibody deficiency, oPAD) and healthy controls (HC) before COVID-19 vaccination and after the second vaccination with the Pfizer-BioNTech COVID-19 vaccine BNT162b2 (Comirnaty). Results for IgG antibodies are expressed as relative units (RE)/ml, the dotted line indicates 11 RE/ml that were used as the cutoff for positivity. Semiquantitative measurements of IgA antibodies are expressed as the ratio between the extinction of patient samples and the extinction of a calibrator provided with the kit; the dotted line indicates a ratio of 1.1 that was considered as the cutoff for positivity. Surrogate virus neutralizing antibodies **(C)** were assessed by testing the ability of serum antibodies (irrespective of isotype) to inhibit the interaction between recombinant SARS-CoV-2 receptor-binding domain and angiotensin-converting enzyme 2 using a blocking ELISA. Results are expressed as percent inhibition; the dotted horizontal line depicts 30% inhibition used as the cutoff for positive SARS-CoV-2 neutralizing antibody. Statistical differences between the two groups depicted in the figure and were determined with a non-parametric two-tailed Mann–Whitney U-test (Kruskal–Wallis H test for all groups: $p < 0.0001$), the median is represented by a horizontal bar.

after COVID-19 vaccination patients with oPAD as a group were comparable to healthy controls with five out of 38 patients showing lower levels of neutralizing antibodies as compared to the control group (**Figure 2C**). Only one out of sixteen patients with a selective IgG antibody deficiency against bacterial polysaccharides failed to produce sVNT antibodies (**Figure 2C**) as well as binding IgG and IgA antibodies against spike protein (**Figures 2A, B**).

Reduced CVID T-Helper Cell Activation After Restimulation With SARS-CoV2 Spike Peptides

PAD patients might benefit from vaccination despite a low or even absent antibody response due to the induction of cellular immunity (24). Activation of both CD4+ T cell subsets was significantly reduced in CVID patients as compared to healthy controls but statistically higher as compared to values obtained in CVID patients before vaccination (**Figures 3A, B**). Patients suffering from oPAD displayed significantly higher percentages of activated cTfh cells after vaccination as compared to vaccinated CVID, but values were still significantly lower than in healthy controls (**Figure 3A**). Activation of CXCR3-negative cTfh, a cTfh subset involved in B cell help for IgG antibody production (20), was significantly reduced in CVID patients as compared to healthy controls (**Figure 3D**), indicating that impaired cTfh activation in CVID affected this cTfh subset known to be crucial for B cell IgG antibody production (20). Impaired CVID cTfh or Tmem response to stimulation with the vaccination antigen could also be observed when T cell stimulation was carried out for a prolonged incubation period (**Figures 3E1, E2**), indicating that activation of CD4-positive T cells in CVID is defective rather than delayed. Defective CVID T cell activation in response to spike peptide stimulation could also be observed when antigen-induced production of TNF-alpha, a cytokine produced very early in antigen-specific T cells following TCR-stimulation (33–35) (**Figure 4A**) or antigen-induced CD69 expression, a cell surface antigen expressed by T cells very early after ligation of the TCR/CD3 complex (36) (**Figure 4B**) were examined. Furthermore, antigen-specific T cell proliferation as a relatively late activation event was also reduced in CVID patients (**Figure 4C**).

Antigen-dependent activation of Tmem was significantly higher for healthy adults and oPAD patients after vaccination as compared to values obtained before vaccination (see **Figure 3B**). The median of the CVID group was the lowest of the three groups, significantly lower than in healthy controls or oPAD patients, but still significantly higher as compared to the median of CVID patients before vaccination (**Figure 3B**). Patients with oPAD showed levels of recall-activation of Tmem that were statistically comparable to healthy controls.

IgG Responsiveness in CVID Patients Following BNT162b2 Vaccination Is Associated With Normal Vaccine-Specific Activation of CXCR5-Negative CD4+ Memory T Cells

BNT162b2-vaccinated CVID patients were grouped into anti-spike IgG-responders and non-responders. The threshold for response

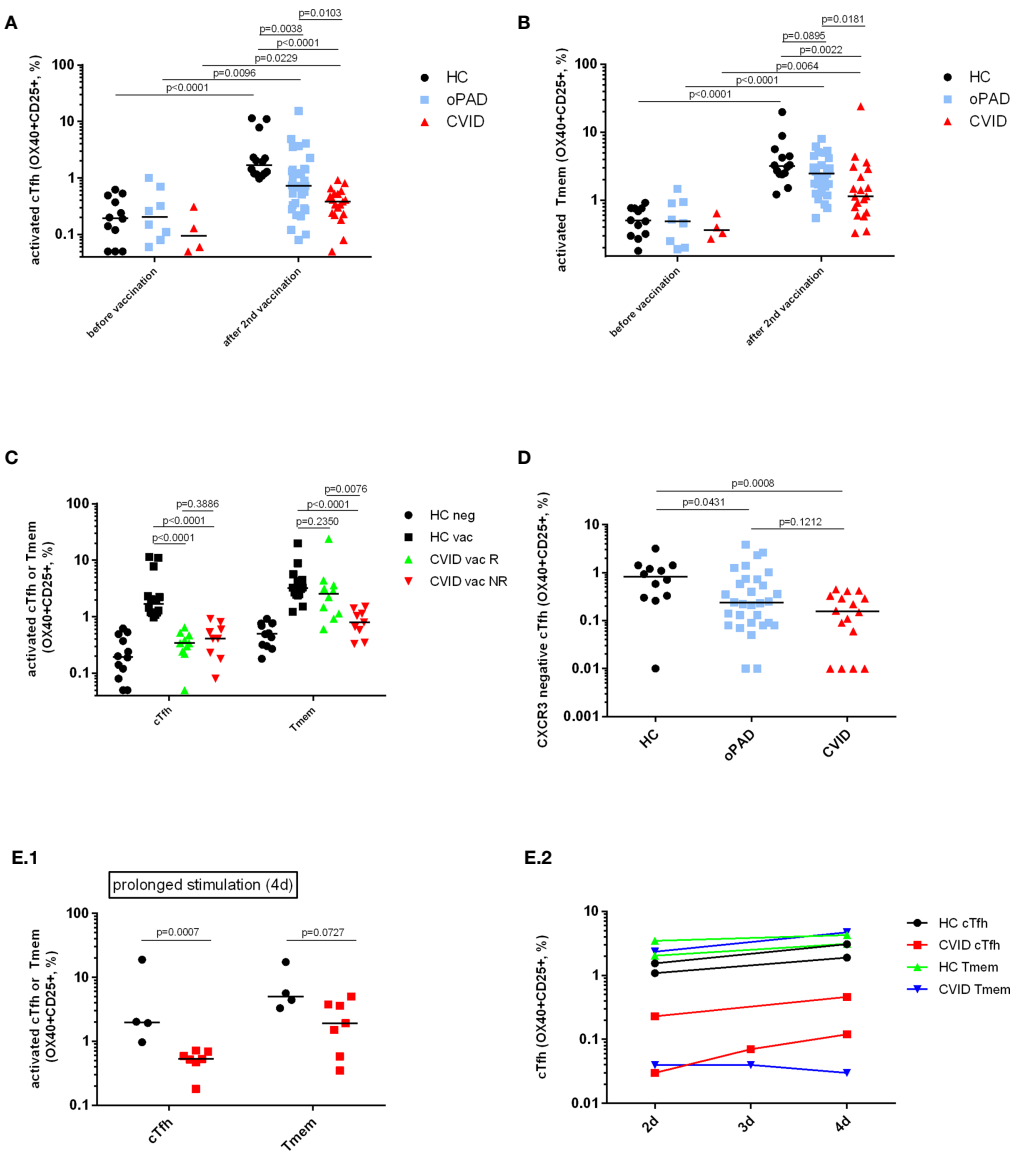

FIGURE 3 | Detection of SARS-CoV-2 spike protein-specific CXCR5$^+$ circulating follicular T-helper cells (cTfh), CXCR3-negative cTfh and CXCR5$^-$ CD4$^+$ T-memory cells (Tmem) by flow cytometry. Human peripheral blood mononuclear cells (PBMC) from healthy controls (HC), CVID patients and patients with other, milder forms of primary antibody deficiency (oPAD), before COVID-19 vaccination and after the second vaccination with BNT162b2, were stimulated for two days using overlapping peptides of immunogenic regions of SARS-CoV2 spike protein (1 µg of peptides/ml). Activation of circulating follicular T-helper cells [CD3$^+$CD4$^+$CD45RA$^-$ CXCR5$^+$, cTfh, panel **(A)**] and CXCR5-negative CD4 memory T cells [Tmem, panel **(B)**] was determined by measuring upregulation of CD25 and CD134 (OX40) by flow cytometry. Results are expressed as percent CD134 (OX40) and CD25 double positive cells relative to the respective CD4$^+$ T cell subpopulation. Unstimulated control cells were incubated in parallel in culture medium only (percent CD134 and CD25 double positive unstimulated cells, median [IQR], before vaccination: HC (n = 12), Tfh 0.04 [0.04], Tmem 0.11 [0.42]; CVID (n = 4), Tfh 0.06 [0.15], Tmem 0.1 [0.2]; oPAD (n = 8), Tfh 0.06 [0.13], Tmem 0.05 [0.01]; after the second vaccination: HC (n = 14), Tfh 0.13 [0.11], Tmem 0.11 [0.42]; CVID (n = 19), Tfh: 0.03 [0.11], Tmem 0.08 [0.14]; oPAD (n = 31), Tfh 0.08 [0.11], Tmem 0.07 [0.53]; Kruskal–Wallis H test: n.s.). Panel **(C)** shows percent of activated, CD134 (OX40) and CD25 double positive cTfh following stimulation of PBMC for two days using overlapping peptides of immunogenic regions of SARS-CoV2 spike protein (1 µg of peptides/ml). PBMC were obtained from healthy controls (HC), CVID responders (anti-spike protein IgG antibody following second vaccination > three times cutoff = 33 RE/ml) and CVID non-responders after the second vaccination against COVID-19. Panel **(D)** shows the percentage of activated CD134 (OX40) and CD25 double positive CXCR3-negative cTfh activated in response to stimulation with SARS-CoV-2 spike peptides. Panel **(E)** shows activation of cTfh and Tmem as assessed by measuring OX40- and CD25-expression following prolonged stimulation with SARS-CoV-2 spike peptides for 4 days [panel **(E.1)**; black circles: healthy controls, red squares:CVID]. In two healthy controls and two CVID patients, cTfh and Tmem activation was examined after two, three and four days of stimulation with SARS-CoV-2 spike peptides [panel **(E.2)**]. Statistical differences between two groups are depicted in the figure and were determined with a non-parametric two-tailed Mann–Whitney U-test (Kruskal–Wallis H test for all groups: p <0.0001), the median is represented by a horizontal bar.

was determined to be 3 times the positive/negative cut-off (cut-off: 11 RE/ml; threshold responder/non-responder: 33 RE/ml of anti-spike IgG). While both CVID subgroups displayed comparably reduced vaccine-specific cTfh responses, only CVID responders displayed vaccine-specific Tmem activation that was statistically comparable to healthy controls. In contrast, CVID non-responders showed decreased levels of Tmem activation that were comparable to healthy controls tested before vaccination (**Figure 3C**). **Figure 1B** shows representative FACS diagrams showing defective antigen-induced activation of both cTfh and Tmem in a CVID IgG antibody nonresponder, while cTfh activation but not Tmem responsiveness is decreased in the CVID responder.

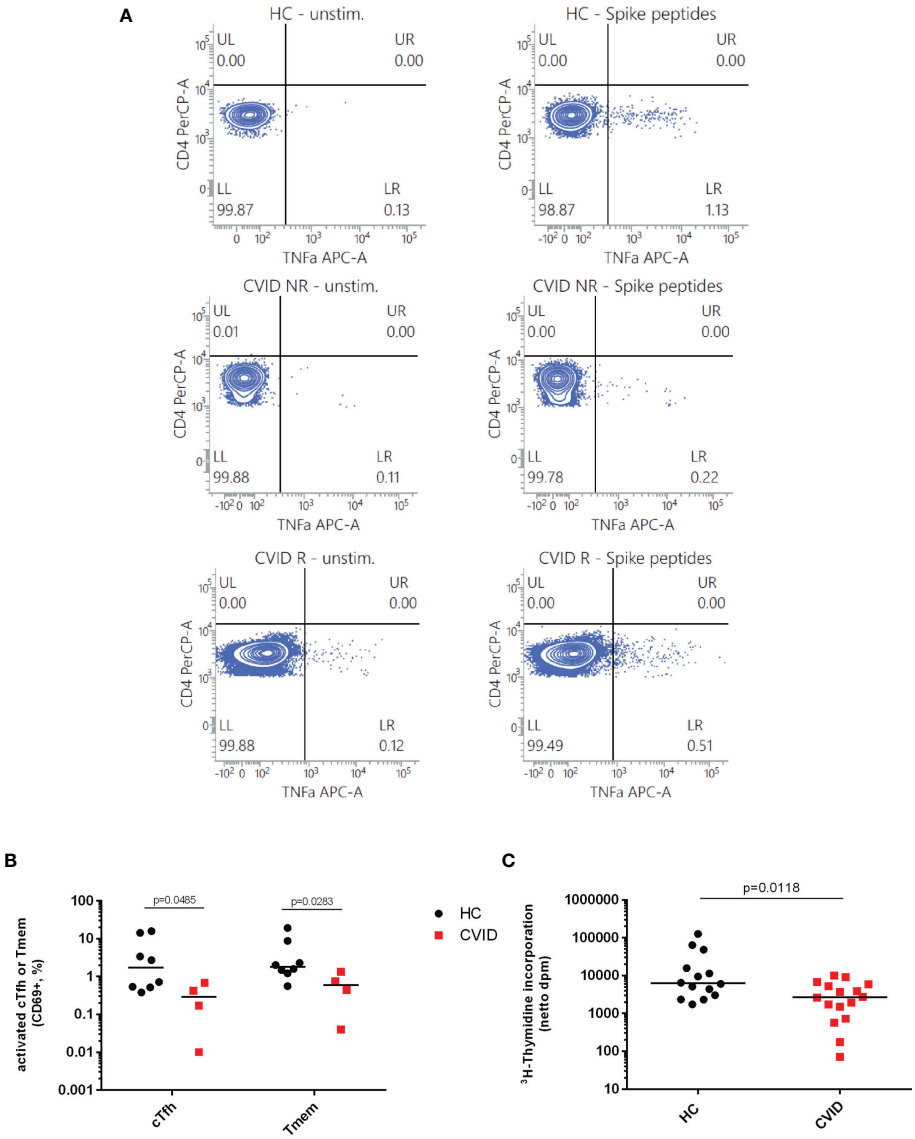

FIGURE 4 | Induction of early activation events such as CD69 expression and TNF-alpha production as well as cell proliferation as a late activation event is reduced in CD4-positive T cells from CVID patients stimulated with SARS-CoV2 spike peptides. TNF-alpha induction was measured as an early activation marker by flow cytometry in $CD3^+CD4^+CD45RA^-$ cells from CVID patients responding with IgG antibody production following BNT162b2 mRNA COVID-19 vaccination (CVID R), CVID nonresponders (CVID NR) and healthy controls (HC). The figures depicted in panel **(A)** are representative for a total number of four CVID patients and three healthy controls investigated. Unstimulated cells incubated in medium alone were examined in parallel. In panel **(B)** CD69 expression was examined by flow cytometry on cTfh and Tmem of CVID and healthy controls stimulated with SARS-CoV-2 spike peptides; median CD69 expression in unstimulated cells incubated in medium alone was 1.25% cTfh and 0.41% Tmem in healthy controls, and 0.66% cTfh and 0.28% Tmem in CVID patients. In panel **(C)**, PBMCs from healthy controls (HC) and CVID patients were stimulated for seven days using SARS-CoV2 Spike peptides before cell proliferation was examined by measuring ^{3}H-thymidine incorporation. Results are expressed as netto dpm of ^{3}H-thymindine incorporation, calculated by subtracting dpm of unstimulated cells from dpm of stimulated cells. In unstimulated PBMCs, ^{3}H-thymidine incorporation [dpm, median (IQR)] was 932.85 (1,838.28) in healthy controls (n = 14) and 185.15 (463.85) in CVID (n = 16). Statistical differences between two groups were determined with a non-parametric two-tailed Mann–Whitney U-test, the median is represented by a horizontal bar.

In addition to normal levels of vaccine antigen-specific Tmem activation, CVID responders had significantly higher levels of serum IgG before immunoglobulin substitution therapy [serum IgG, mg/dl, median (IQR), CVID nonresponders 121 (58), CVID responders 272 (232), p = 0.0116] and a trend towards higher percentages of $CD19^+$ lymphocytes [% $CD19^+$ lymphocytes, median (IQR), CVID nonresponders 7 (6.9), CVID responders 14 (4), p = 0.070] and higher percentages of MZ-like IgM memory cells among the $CD19^+$ B cells [$CD27^+IgD^+$, % of $CD19^+$ lymphocytes, median (IQR) CVID nonresponders 8 (11.4), CVID responders 23 (19), p = 0.0537] as compared to CVID non-responders (**Table 1**). In contrast there was no difference between CVID responders and non-responders with respect to age at diagnosis, age at time of second vaccination against COVID-19, days between second vaccination and testing of immune response, serum IgA and IgM levels, distribution between the sexes, absolute and relative numbers of $CD4^+$ and $CD8^+$ cells in peripheral blood, percentage of switched B memory cells among the $CD19^+$ lymphocytes, the ability of the patients to produce IgG antibodies against vaccination antigens, and lymphoproliferative responses to PHA as examined by measuring ^{3}H-thymidine incorporation (**Table 1**).

T Helper Cells From CVID Patients Show Defective TCR-Mediated Activation Following Stimulation With *Staphylococcus aureus* Enterotoxin B

As recall antigen-induced activation of $CD4^+$ T cells was defective in CVID patients following the second COVID-19 vaccination—with defective Tmem activation correlated to IgG non-responsiveness—we next investigated whether TCR-mediated activation of CVID $CD4^+$ T cells was defective when an antigen-independent stimulus was applied. The results depicted in **Figure 5** indicate that cTfh and Tmem from CVID patients showed significantly reduced levels of activation marker expression in response to SEB stimulation as compared to healthy controls (HC) or oPAD patients, indicating that defective antigen-specific recall activation of cTfh and Tmem following COVID-19 vaccination was associated with a more general defect in TCR-mediated $CD4^+$ T cell activation in CVID patients. Levels of SEB-activated $CD4^+$ T cell subsets in HC and oPAD were comparable (**Figure 5A**). When we analyzed SEB-induced cTfh and Tmem activation in anti-spike IgG-responsive and non-responsive CVID patients, only the IgG-nonresponders showed significantly reduced SEB-mediated activation of cTfh and Tmem as compared to healthy controls (**Figure 5B**), indicating that impaired anti-spike protein IgG response was associated with defective TCR-mediated $CD4^+$ T cell activation.

DISCUSSION

The present study shows that overall 76% of patients with primary antibody deficiency produced protective anti-SARS-CoV-2 IgG antibodies following two doses of the mRNA vaccine BNT162b2, supporting the recommendation that patients with primary antibody deficiency should be vaccinated against COVID-19, preferentially using an mRNA vaccine (https://www.cdc.gov/vaccines/covid-19/clinical-considerations/covid-19-vaccines-us.html). In the different forms of primary antibody deficiency the likelihood of a positive IgG response depends on the severity of the immunological phenotype. Patients with immunoglobulin isotype deficiency and/or selective IgG antibody deficiency against polysaccharide antigens had a largely normal capacity to produce neutralizing antibodies after two vaccinations with BNT162b2 (38/39 = 97.4% were IgG responders). In contrast, patients with CVID have in common a defective IgG production to various antigens, as evaluated by repeated serum immunoglobulin measurements, B-cell enumeration and phenotyping, and diagnostic vaccination (10, 37, 38). Accordingly, more than half of our CVID cohort failed to mount a significant IgG response following BNT162b2 vaccination. Along these lines it is remarkable that in our study as well as in previous reports (18, 19) a significant subgroup of patients with CVID vaccinated with BNT162b2 against SARS-CoV-2 produced vaccine-specific IgG antibodies with levels comparable to healthy controls. The immunoglobulin products our patients received at the time of testing were negative for SARS-CoV-2 spike protein-specific IgG antibodies, and IgG-antibodies against SARS-CoV-2 nucleocapsid protein were negative in all vaccinated patients (data not shown), indicating that the observed anti-spike IgG responses were indeed produced by the patients in response to mRNA vaccination and not passively transferred by ongoing immunoglobulin substitution therapy. While the IgG responses of the CVID patients against other antigens were not reported in the two previous studies (18, 19) it is interesting to note that SARS-CoV-2 IgG responders and non-responders among our CVID patients had comparably defective IgG antibody responses to a variety of other microbial pathogens and vaccination antigens such as staphylococcal or streptococcal toxins, viral antigens such as measles, mumps, rubella or VZV, pneumococcal or Hib capsular polysaccharides, and tetanus or diphtheria toxoids, indicating a potentially increased immunogenicity of mRNA vaccines in CVID patients, thus stimulating IgG antibodies when more conventional forms of antigen delivery failed. IgG antibody responses after vaccination with influenza virus antigen, bacterial polysaccharides or diphtheria and tetanus toxoids in selected CVID patients have been previously associated with milder clinical symptomatology (39), higher levels of switched B memory cells (40) or more circulating plasmablasts (41) as compared to nonresponders. In our study CVID responders showed significantly higher levels of serum IgG before immunoglobulin substitution therapy, and a trend towards higher percentages of $CD19^+$ lymphocytes and higher percentages of MZ-like IgM memory B cells as compared to CVID non-responders, indicating that together with new mRNA vaccine technology less severe impairment of immunity might be responsible for intact IgG responses to BNT162b2 vaccination in these patients.

Most CVID patients also have IgA deficiency (9) and IgA deficiency was recently postulated as a risk factor for developing severe COVID-19 in CVID patients (42). The overall majority (87%) of our CVID patients failed to produce IgA antibodies

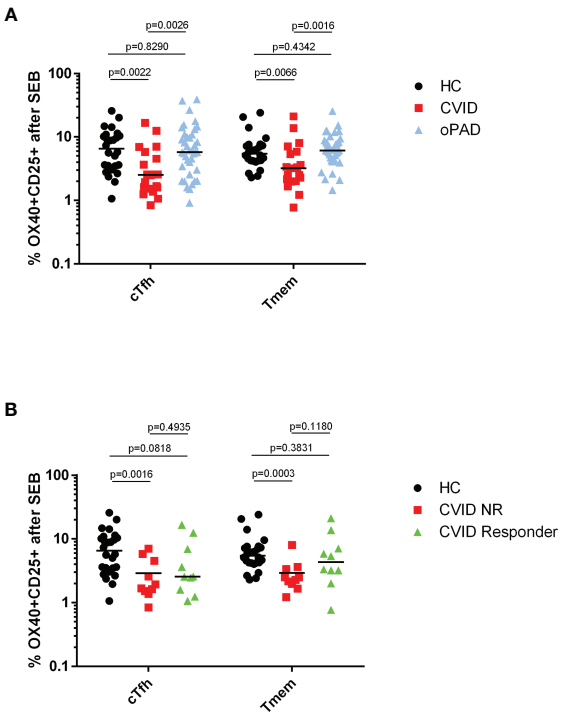

FIGURE 5 | Defective SEB-induced activation of circulating follicular T helper cells and CXCR5-negative CD4 memory T cells from CVID patients. Panel **(A)** human peripheral blood mononuclear cells (PBMC) from healthy controls (HC), CVID patients and patients with other, milder forms of primary antibody deficiency (oPAD) were stimulated for two days with the bacterial superantigen Staphylococcus enterotoxin B (SEB, final concentration 1 µg/ml) before activation of circulating follicular T-helper cells ($CD3^+CD4^+CD45RA^-CXCR5^+$, cTfh) and CXCR5-negative CD4 memory T cells (Tmem) was determined by measuring upregulation of CD25 and CD134 (OX40) with flow cytometry. Results are expressed as percent CD134 (OX40) and CD25 double positive cells relative to the respective $CD4^+$ T cell subpopulation. Panel **(B)** human peripheral blood mononuclear cells (PBMC) from healthy controls (HC), CVID IgG antibody responders (anti-spike protein IgG antibody following second vaccination > three times cutoff = 33 RE(/ml) and CVID IgG antibody non-responders were stimulated for two days with the bacterial superantigen Staphylococcus enterotoxin B (SEB, final concentration 1 µg/ml) before activation of circulating follicular T-helper cells ($CD3^+CD4^+CD45RA^-CXCR5^+$, cTfh) and $CXCR5^-$ CD4 memory T cells (Tmem) was determined by measuring upregulation of CD25 and CD134 (OX40) with flow cytometry. Results are expressed as percent CD134 (OX40) and CD25 double positive cells relative to the respective $CD4^+$ T cell subpopulation. Statistical differences between two groups given in the figure were determined with a non-parametric two-tailed Mann–Whitney U-test (Kruskal–Wallis H test for all groups: $p < 0.0001$), the median is represented by a horizontal bar.

against SARS-CoV-2 spike protein, including even patients who were capable of producing IgG antibodies following BNT162b2 vaccination. In contrast, the IgA response after BNT162b2 vaccination was comparable in patients with milder forms of primary antibody deficiency and healthy controls (**Figure 2B**). Impaired IgA response following vaccination has been described as a prognostic marker in CVID (43), but whether deficient IgA responses in immunized CVID patients are relevant for protection against SARS-CoV-2 infection remains to be determined.

Although the mechanisms leading to BNT162b2 vaccine responsiveness in PAD patients are likely diverse, our results show that a preserved antigen-specific $CD4^+$ T memory cell response might play an essential role. CXCR5-positive Tfh cells are known to be important for the formation of germinal centers, B cell proliferation, antibody diversification and affinity maturation, isotype switching and the differentiation of B cells into memory cells and antibody-secreting plasma cells (20, 23), and antibody nonresponsiveness to H1N1 influenza virus vaccine has been correlated with altered vaccine-specific Tfh responses in immunocompromised populations (21). In our study impaired activation of CXCR5-negative $CD4^+$ memory cells but not cTfh characterized IgG nonresponsiveness in patients with CVID, while antigen-specific activation of CXCR5-positive cTfh was comparably reduced in CVID BNT162b2-IgG-responders and nonresponders. The mechanism whereby CXCR5-negative $CD4^-$ T cells might influence B cell responses remains to be determined. Of particular interest in this respect are the recently described CXCR5-negative human peripheral helper T cells which have first been shown to expand in autoimmune diseases (44). Up to now participation of CXCR5-negative peripheral helper T cells in B cell responses has been limited to autoantibody and alloantibody production (44, 45), but these helper T cells could also play a role in mRNA vaccine-specific antibody responses. Both T helper subsets exert B helper activities using comparable mechanisms to some extent, e.g., *via* IL-21 expression (46) and baseline levels of CXCR5-negative peripheral helper T cells strongly predict the ability to produce anti-spike RBD IgG antibodies in immunocompromised transplant patients vaccinated against COVID-19 (47). Further studies are needed to confirm that CXCR5-negative peripheral helper T cells induce B cell responses in CVID patients following COVID-19 vaccination, but it is intriguing to note that peripheral T helper cells have been implicated in extrafollicular B cell differentiation (46), and IgG responses in CVID patients vaccinated against COVID-19 have been ascribed to atypical memory B cell responses producing low affinity spike protein-specific antibodies as a result of extrafollicular B cell differentiation (18). The emergence of extrafollicular B cell responses during symptomatic COVID-19 has been recently described, thus potentially disrupting the normal follicular B cell differentiation known to result in long-term protection (48). Although our results suggest that the IgG antibodies produced by CVID responders with the help of "atypical" CD4 helper cells are functional shortly after vaccination, it remains to be determined whether CVID responders mount a booster response following subsequent vaccination that is required for long-term protection, in particular against new variants of SARS-CoV-2.

Our study shows that activation of cTfh in response to stimulation with a vaccination antigen is defective in CVID, and that prolonged stimulation with the antigen does not restore T cell activation, indicating that CD4 T cell activation is not merely delayed in CVID (**Figure 3E**). Abnormalities in $CD4^+$ T cell activation could be responsible for impaired antigen-specific

$CD4^+$ memory cell activation leading to IgG non-responsiveness, as TCR-mediated activation of $CD4^+$ T cells after SEB stimulation was defective in CVID BNT162b2 IgG nonresponders. Defective TCR-mediated signalling has been previously described in CVID T cells (30) and could play a role in IgG non-responsiveness, as inhibition of TCR-mediated Tfh cell activation has been shown to impair antibody responses and T cell help for immunoglobulin production *in vitro* (49). Defective TCR-mediated T cell activation in CVID could affect Tfh more severely than other types of CD4-positive T cells such as Tmem. Along these lines it has been described that activation of Tfh requires particularly strong and sustained TCR/ligand interactions (50), activation requirements that could be affected by the costimulation defect previously shown to lead to defective TCR-dependent T cell activation in CVID (30).

A major limitation of our study is that we examined vaccination responses at one time point only, as delayed responses to SARS-CoV-2 vaccination have been reported in immunocompromised populations (51, 52). Longevity and functionality of B cell responses after vaccination depend on intact $CD4^+$ T cell responses, in particular of the Tfh subset (23). It remains to be determined whether decreased Tfh responses in PAD as compared to healthy controls lead to a more rapid decline of IgG antibody titers and/or impaired booster responses. Alternatively additional booster immunizations could lead to enhancement of SARS-CoV-2 cTfh and antibody responses, as intact Tfh responses have been reported in CVID following vaccination against influenza, an antigen repeatedly encountered in adult life (16).

The anti-spike IgG antibodies produced by PAD patients in our study were functional in a surrogate virus neutralization test, as has also been reported previously (19), which is in contrast to the previous observation that memory B cells with high specificity against the receptor binding domain of the viral spike protein, cells known to produce most of the neutralizing antibodies against SARS-CoV-2, are undetectable in COVID-19 vaccinated CVID patients (18). The diagnosis of CVID is made by exclusion in at least 90% of the patients, which leads to the considerable immunological, clinical and genetic heterogeneity of patients that might explain some of the discrepancies between our results and previous studies (18, 19). Our findings are nevertheless very encouraging with respect to the question whether patients with PAD should be vaccinated against COVID as even a subgroup of CVID patients that were deficient in IgG responses against various other antigens produced significant levels of functional anti-spike IgG antibodies that should confer protection against infection with SARS-CoV-2 to this vulnerable population.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/snp/ accession numbers rs1131690788 and rs1724867456.

ETHICS STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and fulfils the guidelines of the Austrian Agency of Research Integrity (OeAWI). With respect to the clinical immunological analyses this study was approved by the Ethics Committee of the Immunology Outpatient Clinic as a study using the biobank of residual specimen of the Immunology Outpatient Clinic. According to the Ethics Committee of the City of Vienna and the legal regulations to be applied (§15a Abs. 3a Wiener Krankenanstaltengesetz) no additional ethics committee evaluation is required for a non-interventional study using data collected as part of the routine medical care the patients received. The patients gave their informed consent that anonymized data collected as part of the routine medical attendance (serum antibody and flow cytometry analysis, activation-induced marker assay) could be included in a scientific publication. All patient information in this study is anonymized and deidentified. No extra intervention was carried out.

AUTHOR CONTRIBUTIONS

KS, CG, and RS performed the T cell activation assays and interpreted the respective results. HA performed the antibody measurements. KS created the figures and tables. KS and HW interpreted and analyzed the overall results, took overall responsibility for the research performed in this study and actively wrote the manuscript. PI, JW, CG, and HW cared for the patients and provided samples. Critical revision of the article was done by ME, CG, and MF. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CASE REPORT
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Case Report: Cytomegalovirus Disease Is an Under-Recognized Contributor to Morbidity and Mortality in Common Variable Immunodeficiency

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Background: Common Variable Immunodeficiency (CVID) is classified as a 'Predominantly Antibody Deficiency' (PAD), but there is emerging evidence of cellular immunodeficiency in a subset of patients. This evidence includes CVID patients diagnosed with cytomegalovirus (CMV) infection, a hallmark of 'combined immunodeficiency'. CMV infection also has the potential to drive immune dysregulation contributing to significant morbidity and mortality in CVID. We aim to determine the extent of cellular immune dysfunction in CVID patients, and whether this correlates with CMV infection status.

Methods: We conducted a single-center retrospective cohort study of individuals with CVID at the Royal Melbourne Hospital, and identified patients with and without CMV disease or viraemia. We then isolated T-cells from patient and healthy donor blood samples and examined T-cell proliferation and function.

Results: Six patients (7.6%, 6/79) had either CMV disease (pneumonitis or gastrointestinal disease), or symptomatic CMV viraemia. A high mortality rate in the cohort of patients with CVID and CMV disease was observed, with 4 deaths in the period of analysis (66.6%, 4/6). Individuals with CMV infection showed reduced T-cell division in response to T-cell receptor (TCR) stimulation when compared with CMV-negative patients.

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Discussion: This study demonstrates the morbidity and mortality associated with CMV in CVID, and highlights the need for focused interventions for patients with CVID at risk of CMV disease.

Keywords: cytomegalovirus, herpesvirus 6, common variable immunodeficiency, predominantly antibody deficiency, primary immunodeficiencies, immunogenetics, cellular immunity

INTRODUCTION

Predominantly Antibody Deficiency (PAD) is the most common Primary Immunodeficiency (PID) diagnosed in adults. The most prevalent PAD is Common Variable Immunodeficiency (CVID), a phenotypically heterogeneous disease characterised by hypogammaglobulinaemia, impaired vaccine responses and recurrent sinopulmonary infections (1). In addition to immunodeficiency, most individuals with CVID (>70%) also display features of chronic immune dysregulation, such as autoimmunity, malignancy and/or autoinflammation (2). These non-infectious manifestations are exceptionally challenging to manage in the context of underlying immunodeficiency.

Diagnosis of CVID typically focuses on confirming an impaired humoral response. However, emerging evidence of cellular immunodeficiency in individuals with CVID has prompted reassessment of this diagnostic framework. Reduced numbers and/or proportions of naïve $CD4^+$ T-cells have been suggested as potential immunophenotypic markers for late-onset combined immunodeficiency, or, 'LOCID' (3–5). Viral infection is another clinically relevant hallmark of cellular immunodeficiency that has diagnostic potential in the context of CVID.

Cytomegalovirus (CMV) is a human beta-herpes virus, with high seroprevalence (40–90%) in the general adult population (6). Initial CMV infection is typically controlled, but persistent viral DNA is detectable within latent reservoirs established in undifferentiated myeloid cells (7). Viral clearance and establishment of latency occurs independent of the humoral immune response; as demonstrated by CMV infection being controlled in murine models of absolute B-cell deficiency (8). Latent CMV, once established, is also predominantly controlled by T-cell immunity (7).

CMV disease is generally defined as the presence of tissue-invasive CMV associated with an appropriate clinical syndrome (7, 9). It occurs when viral replication is reactivated, and is commonly encountered in secondary immunodeficiencies, for example, following haematopoietic stem cell transplantation (HSCT), or in the setting of acquired immunodeficiency syndrome. It was recently reported that CMV disease in CVID is relatively common and can result in fatal clinical outcomes (9). However, knowledge of CMV's impact on the immune response in the context of CVID-associated immunodeficiency is limited.

A previous study examining T-cell responses in CVID patients with evidence of CMV exposure suggested that aberrant immune responses to CMV may directly cause inflammatory dysregulation in CVID (10). Here, an expanded population of CMV-specific late-memory T cells ($CD8^+/CD27^-/CD28^-$) was observed, as well as an increased production of pro-inflammatory cytokines in response to CMV antigens in CVID patients with a history of CMV infection (10–12).

However, there is a lack of evidence regarding factors that precipitate CMV disease (as opposed to asymptomatic viraemia or latent infection) in CVID. Research to date mainly comprises of case reports (13–35), while larger-scale studies have included secondary immunodeficiencies (36) or focused on CMV in the setting of iatrogenic immunosuppression (37).

With the increasing use of HSCT as a curative treatment for adult PIDs, there is a pressing need to better understand the specific immune deficiencies that lead to loss of latency and development of CMV disease in the context of CVID; CMV seroprevalence is common, and active CMV disease presents a contraindication to transplant. To address this knowledge gap, we conducted a single-center study of CMV disease in CVID and explored its possible association with genetic diagnosis, T-cell proliferation and function.

METHODS

Subject Selection

A retrospective cohort study of individuals under the care of The Royal Melbourne Hospital Clinical Immunology & Allergy Unit from 2016 to 2021 was conducted, with potential participants identified through internal auditing. The diagnosis of CVID was subsequently confirmed according to European Society for Immunodeficiencies (ESID) criteria (1). Medical records and pathology from 2016 to 2021 were interrogated for the diagnosis of CMV disease (histological evidence of tissue-invasive CMV, associated with an appropriate clinical syndrome) or symptomatic viraemia ('CMV syndrome'), as well as factors associated with CMV infection (including lymphocyte subsets, inflammatory complications of CVID, subsequent immunosuppression, and infection history).

For functional studies, individuals with CVID and no history of clinical CMV ($CVID^+/CMV^-$) were recruited as 'controls' for patients with CVID and clinical CMV ($CVID^+/CMV^+$). Healthy donors ($CVID^-/CMV^-$) with no significant past medical history were enrolled through the Volunteer Blood Donor Registry (WEHI).

Preparation of Lymphocytes and T-Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood by density gradient Ficoll-Leucosep centrifugation. Cells were frozen and cryopreserved in liquid nitrogen. T cells were isolated *via* negative selection using a Human T-cell Isolation Kit

(Stemcell Technologies, Vancouver, Canada) following the manufacturer's instructions. Purity of isolated $CD3^+$ T-cells was >99%.

Antibodies and Dyes

CD3-V500 (clone UCHT1) and CD4-APC (clone RPA-T4) were purchased from BD Pharmingen, San Jose, California. CD8-APC780 (clone RPA-T8), CD45RA-PeCy7 (clone HI100) and CD45RO-PE (clone UCHL1) were purchased from eBioscience, San Diego, California. CD27-FITC (clone M-T271) was purchased from Miltenyi Biotec, Bergisch Gladbach, Germany. Dead cells were excluded from analysis using propidium iodide (PI) (Sigma-Aldrich, St. Louis, Missouri). All antibody cocktails were made using Brilliant Stain Buffer (Becton Dickinson, Franklin Lakes, New Jersey).

T-Cell Proliferation Assay

Purified T cells were labelled with CellTrace Violet (Thermo Fisher Scientific Australia, Scoresby, Australia) (38), plated in triplicate (1×10^4 cells/well) and incubated for 96 hours at 37°C in the presence of: 400 U/mL IL-2 (Abcam, Boston, Massachusetts), 1 bead/cell Human T-Activator CD3/28 Dynabeads (Thermo Fisher Scientific Australia, Scoresby, Australia), or 1x PHA (Thermo Fisher Scientific Australia, Scoresby, Australia) as indicated. Data on cell counts and proportions were collected using a BD FACSCanto Clinical Flow Cytometer every 24 hours for 4 days.

Data were analyzed using FlowJo software, version 10 (Tree Star, Ashland, Oregon). The gating strategy is illustrated in **Supplementary Material (Figure X)**.

Cytokine Assays

T cells were stimulated as described above and supernatant harvested after 48 hours, for all conditions. Quantification of IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33 was performed using the LEGENDplex™ Human Inflammation Panel 1 (BioLegend, San Diego, California), according to the manufacturer's instructions. Quantification of IL-2 was performed using the V-PLEX Human IL-2 kit (Meso Scale Discovery, Rockville, Maryland).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software, version 9 (GraphPad Software, Inc, La Jolla, California). For binary outcomes, cohorts were compared using Fisher's exact tests due to the small sample sizes. For continuous variables, Kruskal-Wallis testing was used for multiple comparisons between cohorts, with the assumption of non-parametric data distribution. Results are shown as means and error bars represent standard errors of the mean (SEM). Two-tailed P values are reported, with values of <0.05 considered statistically significant.

Ethics

Ethical approval for the study protocol was granted by the Human Research Ethics Committees of Melbourne Health (project reference number 2009.162) and WEHI (project reference number 10/02). Written, informed consent was obtained from all participants, in accordance with the Declaration of Helsinki and subsequent amendments. For individuals who were deceased at the time of data collection, ethical approval was obtained to review their medical records.

RESULTS

Clinical and Immunological Features of CVID Patients With CMV Disease

Our cohort consisted of 79 individuals with CVID. Ten patients (12.7%) had CMV Polymerase Chain Reaction (PCR) testing measured during the period of study, performed where there was clinical suspicion of CMV disease; asymptomatic CMV screening is not part of routine care at our center. Six patients (7.6%) had current or historical evidence of CMV disease or symptomatic viraemia (**Table 1**). The male: female ratio was 2:1, with an age range of 31 to 60 years. Three patients were recruited for functional immunological assessment: Patient 1 (32M), Patient 2 (58F) and Patient 5 (58M).

Three $CVID^+/CMV^-$ patients were selected for comparison, on the basis of age-matching (Patient 7, 35M and Patient 8, 62F), or the presence of an identical underlying monogenic defect (Patient 9, 33F, daughter of Patient 2). Clinical and immunological data on all $CVID^+/CMV^+$ and $CVID^+/CMV^-$ patients are available in **Tables 1**, **2** and **Supplementary Material (Tables 1S, 2S)**. Functional immunological analysis was undertaken on two healthy donors: a 31-year-old male and 58-year-old male (CMV serostatus not known).

Rates of end-organ manifestations in the $CVID^+/CMV^+$ patients were as follows: CMV colitis/enterocolitis 83.3% (5/6), CMV pneumonitis 50% (3/6) and symptomatic viraemia 33.3% (2/6) add TACI mutation. A likely monogenic cause of CVID was identified in 66.7% (4/6), and the remaining 2 patients carried either a CVID risk gene (TNFRSF13B; P.Cys104arg variant) or variant of uncertain significance (ZAP70; heterozygous for c.512A>G).

All $CVID^+/CMV^+$ patients (100%, 6/6) had inflammatory manifestations of disease – for example, autoimmune cytopenias, inflammatory arthropathy, or lymphocytic colitis – with subsequent iatrogenic immunosuppression that preceded the diagnosis of CMV disease. In comparison, across the unit's CMV-negative CVID cohort (n=73), the prevalence of inflammatory disease was 67.1% (49/73, p=0.0923) and the prevalence of immunosuppression exposure was 23.3% (17/73, $p<0.0001$).

Peak viral loads ranged from 113 to 435,757 copies/mL. Higher viral loads did not demonstrate clear association with severity of infection. However, it is possible that 'true peaks' were missed, as surveillance of CMV viral loads was not routinely performed. All patients were treated with anti-viral therapy (**Table 1**). None developed viral resistance, but two patients (Patient 3 and Patient 5) were unable to complete therapy (ganciclovir/valganciclovir) due to profound neutropaenia. Two patients required salvage therapy using adoptive CMV-specific T-cell therapy (Patient 4) and CMV-specific immunoglobulin (Patient

TABLE 1 | CVID^{+}/CMV^{+} cohort: clinical characteristics.

PATIENT	AGE/ GENDER	GENETIC DIAGNOSIS	AGE OF CVID SYMPTON ONSET	AGEAT CVID DIAGNOSIS	AGE AT CMV DIAGNOSIS (YEARS FROM CVID Dx)	INFLAMMATORY DISEASE[#]	IATROGENIC IMMUNOSUPPRESSION (YEAR ADMINISTERED)	CMV MANIFESTATION: YEAR OF DIAGNOSIS (MODE OF DIAGNOSIS)	CMV TREATMENT^	OTHER INFECTIONS	OUTCOME
1	32M	p50 haploinsufficiency (*NFKB1*); pathogenic	10	26	30 (+4)	AIHA, inflammatory arthropathy, lymphocytic enteropathy, non-cirrhotic portal hypertension	Rituximab & prednisolone (2017-2019) Adalimumab (2021)	Enteritis: 2018 (IHC & tissue PCR), Pneumonitis, 2021 (BAL PCR)	G, V	Chronic *Helicobacter Pylori*, pulmonary Aspergillosis, recurrent sinopulmonary infections	Recurrent disease Deceased 2021
2	58F	p50 haploinsufficiency (*NFKB1*); pathogenic	30	38	52 (+14)	Autoimmune pancytopenia, non-cirrhotic portal hypertension: liver transplant	Everolimus, prednisolone & cyclosporin (2016-2021)	Symptomatic viraemia: 2017, 2019 (whole blood PCR)	G, V	Chronic Norovirus, recurrent *Campylobacter*, recurrent sinopulmonary infections	Treatment success, chronic viraemia
3	31M	CTLA4 haploinsufficiency; pathogenic	16	23	17 (-6)	Severe lymhocytic enteropathy, autoimmune pancytopaenia, Burkitt's lymphoma	Hyper-CVAD (2007) Rituximab (2007) Abatacept (2019-2020)	Enteritis: 2012, 2020 (IHC & tissue PCR), Pneumonitis: 2020 (BAL cytology & PCR), Chronic asymptomatic viraemia: 2016-2021 (whole blood PCR)	G, V, *V-induced neutropaenia*	Chronic Norovirus, recurrent sinopulmonary infections	Recurrent disease Deceased 2021
4	60F	*TNFRSF13B* variant; risk gene	37	42	58 (+16)	Granulomatous lymphocytic interstitial lung disease – lung transplant	Rituximab & azathioprine (2017) Prednisolone & tacrolimus (2017-2019)	Enteritis: 2019 (IHC & tissue PCR)	G, V, CMVIg^{+} Lifelong suppressive V	Recurrent sinopulmonary infections	Viral suppression Deceased 2019
5	58M	*ZAP70* heterozygous; variant of uncertain significance	46	50	52 (+2)	AIHA, inflammatory colitis, seronegative spondyloarthropathy	Rituximab & prednisolone (2012, 2014, 2020)	Enteritis: 2014, 2020 (IHC & tissue PCR)	G, V, CMV TCs *G-induced neutropaenia*	Oral candidiasis, recurrent Gram-negative sepsis	Recurrent disease
6	32M	IκBNS deficiency (*NFKBID*); likely pathogenic	15	19	23 (+4)	Pauci-immune crescenteric glomerulonephritis, non-cirrhotic portal hypertension, autoimmune pancytopaenia	Prednisolone (2016) Tocilizumab (2018)	Enteritis: 2018 (IHC & tissue PCR), Pneumonitis: 2018 (BAL cytology & PCR), Chronic symptomatic viraemia: 2016-2021 (whole blood PCR)	G, V	Epstein-Barr viral hepatitis, oral candidiasis	Refractory viraemia Deceased 2019

[#]Inflammatory disease defined as the presence of autoimmune cytopaenia, autoimmune haemolytic anaemia (AIHA), enteropathy, lymphadenopathy/splenomegaly, lymphoproliferative disease, interstitial lung disease or seronegative spondyloarthritis^G, ganciclovir; V, valganciclovir; CMVIg, CMV-specific immunoglobulin; CMV TCs, Adoptive CMV-specific T lymphocyte therapy; $^{+}$Cytogam (CSL Behring), 150 mg/kg, two infusions.

TABLE 2 | CVID$^+$/CMV$^+$ cohort: immunological characteristics.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Peak CMV titre, copies/mL	2,457	18,445	1,380	113	2,382	435,757
Total lymphocyte count, cells x 10^9, [1.0-4.8]*	1.5	1.1	2.0	1.5	1.1	**0.1**
CD19$^+$, cells x 10^9, [0.07-0.55]	0.07	0.11	**0.04**	0.4	0.18	**0.00**
CD3$^+$, cells x 10^9, [0.60-2.50]	1.31	0.89	1.87	0.99	0.88	**0.07**
CD4$^+$, cells x 10^9, [0.45-1.70]	0.5	0.51	**0.42**	0.79	0.58	**0.06**
CD8$^+$, cells x 10^9, [0.20-1.15]	0.72	0.35	1.42	**0.16**	0.27	**0.01**
CD4:CD8, [1.1-2.4]	**0.69**	1.46	**0.3**	**4.94**	2.15	**6**
CD56$^+$, cells x 10^9, [0.07-0.70]	0.12	0.1	0.08	0.11	0.07	**0.02**
Immunoglobulin Replacement Therapy	Intragam 10, 45g q3w (0.66 g/kg/month)	Intragam 10, 25g q2w (0.83 g/kg/month)	Intragam 10, 47.5g q42 (0.79 g/kg/month)	Intragam 10, 45g q4w (0.75 g/kg/month)	Intragam 10, 40g q4w (0.55 g/kg/month)	Intragam 10, 25g q2w (0.66 g/kg/month)
Trough IgG at time of CMV diagnosis, g/L	7.1 (2021) 6.0 (2018)	10.0 (2019) 13.6 (2017)	4.6 (2020)	8.6 (2019)	8.1 (2020)	6.9 (2018)

**[reference range]; values outside reference range in bold.*

5). Patient 4 achieved viral suppression, but Patient 5 continues to have recurrent CMV disease.

All CVID$^+$/CMV$^+$ patients were on intravenous immunoglobulin replacement with physiological Immunoglobulin G (IgG) trough levels (**Table 2**), but had high rates of active bacterial infection. Each of the six patients required, on average, >2 courses of oral antibiotics annually for sinopulmonary infection, and five patients (5/6, 66.7%) were hospitalized ≥ once/year with infection over the period of study. Five of the CVID$^+$/CMV$^+$ cohort (5/6, 83.3%) also had a history of other opportunistic infections: chronic Norovirus, Epstein-Barr virus, chronic candidiasis and Aspergillosis. In contrast, 8.21% of the centre's CMV-negative CVID cohort (6/73) had a history of other disseminated viral infections: varicella-zoster virus, human papillomavirus and Epstein-Barr virus.

There were 4 deaths in the CVID$^+$/CMV$^+$ group over the period studied. These mortalities constituted 66.7% (4/6 deaths in our cohort of 79 individuals with CVID) of unit mortalities over that time, suggesting that CMV disease increased the relative risk of death by 26.3 in individuals with CVID (Fisher's exact test, CI 6.31-102.9; p<0.0001). Only one death was directly attributable to a complication of CMV disease (Patient 1, who died of septic shock secondary to CMV pneumonitis and pulmonary Aspergillosis). Three of the 4 patients (75%) in the CVID$^+$/CMV$^+$ cohort had a flare of CMV disease in the 6 months prior to death, presenting a significant barrier to immunomodulatory treatment of their inflammatory disease.

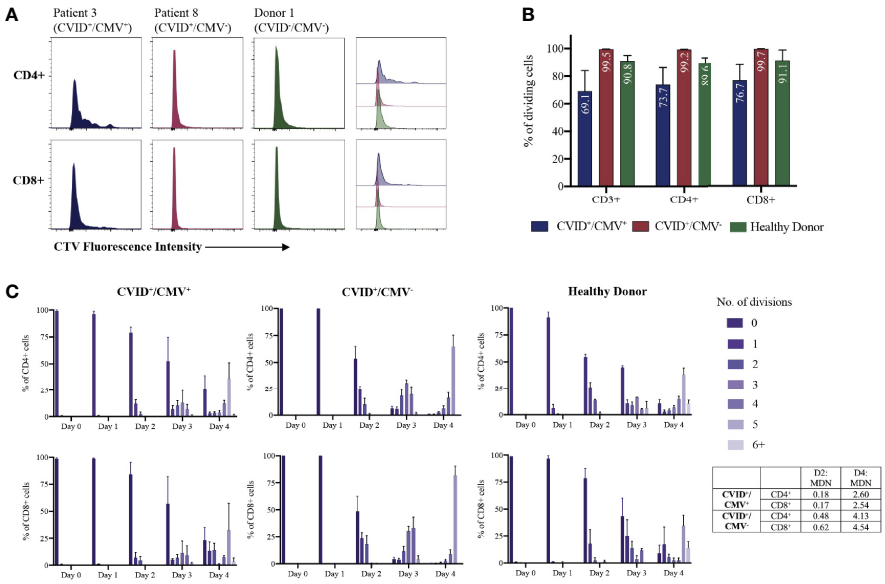
Immunophenotyping of major lymphocyte subsets (**Table 2**) revealed lymphocyte counts ≤ 2.0 x 10^9 cells in all patients, but severe lymphopaenia only in Patient 6. One third (2/6) of patients had reduced (lower than the standard reference range for age) numbers of CD19$^+$ total B cells, 16.6% (1/6) had reduced numbers of CD3$^+$ T cells, 33.3% (2/6) had reduced numbers of CD4$^+$ T cells, 33.3% (2/6) had reduced numbers of CD8$^+$ T cells and 16.6% (1/6) had reduced numbers of CD56$^+$ NK cells. The CD4:CD8 ratio was reduced in 33.3% (2/6) and elevated in 33.3% (2/6). Historical T cell immunophenotyping was available for three individuals. Naïve CD4$^+$ T cells constituted 14.4% of total CD4$^+$ T cells for Patient 1, 15.3% of total CD4$^+$ T cells for Patient 4 and 6.74% of total CD4$^+$ T cells for Patient 5, therefore only one of these patients met the Frieburg immunophenotypic criteria for combined immunodeficiency (naïve CD4$^+$ T cells <10%) (5).

Delayed Proliferative Potential of CVID+/CMV+ T Cell Populations

We next investigated T cell proliferative responses and cytokine production in CVID$^+$/CMV$^+$ patients where possible (P1, P2 and P5), compared to matched CVID$^+$/CMV$^-$ patients (P7, P8, P9) and healthy donors. Total CD3$^+$ T cells were isolated from each group, labelled with division tracking dye CTV, stimulated with IL-2, CD3/CD28, IL-2 + CD3/CD28 or PHA, harvested daily for 4 days and the proliferative potential assessed through analysis of CTV fluorescence intensity (**Figures 1A, B**).

Delayed CD3$^+$ proliferation in response to IL-2 + CD3/28 stimulation was observed in the CVID$^+$/CMV$^+$ cohort (**Figure 1C**). On average, 78.9% of CD4$^+$ T cells and 84.1% of CD8$^+$ T cells in the CVID$^+$/CMV$^+$ group remained undivided at 48 hours, compared with 53.6% of CD4$^+$ T cells and 48.5% of CD8$^+$ T cells in the CVID$^+$/CMV$^-$ group. At the final time point (Day 4), a reduced mean proportion of T cells had undergone division (69.1%) in the CVID$^+$/CMV$^+$ cohort, compared to 99.5% in the CVID$^+$/CMV$^-$ cohort and 90.8% in healthy donors (**Figure 1B**). CVID$^+$/CMV$^+$ T-cells underwent, on average, fewer rounds of cell division (mean division number, MDN) than CVID patients without CMV, or healthy donors (Day 4 CD4$^+$ MDN: 2.60 in CVID$^+$/CMV$^+$, 4.13 in CVID$^+$/CMV$^-$, 3.42 in healthy donors, Day 4 CD8$^+$ MDN: 2.54 in CVID$^+$/CMV$^+$, 4.54 in CVID$^+$/CMV$^-$, 3.01 in healthy donors).

T-cell proliferation to PHA stimulation was diminished in both the CVID$^+$/CMV$^+$ and CVID$^+$/CMV$^-$ groups compared to healthy donors. The mean proportion of divided T cells at day 4



		D2: MDN	D4: MDN
CVID+/CMV+	CD4+	0.18	2.60
	CD8+	0.17	2.54
CVID+/CMV-	CD4+	0.48	4.13
	CD8+	0.62	4.54

FIGURE 1 | T-cell proliferation to IL-2 + CD3/28 stimulation. **(A)** Individual examples of $CD4^+$ and $CD8^+$ T-cell proliferation at Day 4, determined by CellTrace Violet (CTV) dilution. **(B)** Proportions of dividing cells at Day 4 for each cohort, expressed as a percentage of total $CD3^+$, $CD4^+$ and $CD8^+$ T-cells. Results presented as means and standard errors of the mean (SEM). **(C)** T-cell proliferation over time: number of $CD4^+$ and $CD8^+$ divisions at each time-point, expressed as a percentage of total $CD4^+$ and $CD8^+$ T-cells. Results presented as means and SEM for each cohort. MDN, Mean Division Number.

was 44.5% in the $CVID^+/CMV^+$ cohort, 35.3% in the $CVID^+/CMV^-$ cohort and 95.6% in healthy donors (**Supplementary Material, Figure Y**). Proportions of divided $CD4^+$ cells were similar in the $CVID^+/CMV^+$ and $CVID^+/CMV^-$ groups (mean of 44.6% in $CVID^+/CMV^+$ and 44.1% in $CVID^+/CMV^-$). There was an increased proportion of divided $CD8^+$ cells in the $CVID^+/CMV^+$ cohort (mean 45.5%) compared with the $CVID^+/CMV^-$ group (mean 20.4%), however results in this group were highly heterogenous (range of undivided $CD8^+$ cells in the $CVID^+/CMV^+$ cohort 24.0-88.5%).

Production of Inflammatory Cytokines by CVID+/CMV+ T Cells

Analysis of the supernatant from proliferating $CD3^+$ T cells (**Figure 2** and **Supplementary Material, Figure Z**) largely demonstrated reduced cytokine generation in both the $CVID^+/CMV^+$ and $CVID^+/CMV^-$ cohorts in comparison to healthy donors, with the exceptions of IL-1β production in the context of IL-2 stimulation, and IFN-γ production following CD3/28 stimulation.

IFN-γ generation by $CD3^+$ T-cells was highest in the $CVID^+/CMV^-$ cohort with CD3/28 stimulation (mean IFN-γ concentration 1739.1 pg/mL in the $CVID^+/CMV^-$ group, vs. 489.5 pg/mL in the $CVID^+/CMV^+$ group and 984.6 pg/mL in healthy donors), but results in this group were heavily skewed by Patient 7, who had a mean IFN-γ concentration of 3922.8 pg/mL. IL-2 + CD3/28 induced IFN-γ production was reduced in both CVID groups (289.8 pg/mL in $CVID^+/CMV^+$, 389.9 pg/mL in $CVID^+/CMV^-$) compared to healthy donors (981.1 pg/mL).

Concentrations of TNF-α, IL-2, IL-6 and IL-17A were highest in healthy donors in the presence of CD3/28 stimulation. Under these conditions, the mean concentration of TNF-α generated by $CD3^+$ T cells in healthy controls was 2346.9 pg/mL (vs. 411.3 pg/mL in $CVID^+/CMV^+$ and 213.4 pg/mL in $CVID^+/CMV^-$), the mean concentration of IL-2 was 12898.9 pg/mL (vs. 3688.3 pg/mL in $CVID^+/CMV^+$ and 3623.0 pg/mL in $CVID^+/CMV^-$), the mean concentration of IL-6 was 40.26 pg/mL (vs. 2.78 pg/mL in $CVID^+/CMV^+$ and 12.73 pg/mL in $CVID^+/CMV^-$) and the mean concentration of IL-17A was 84.74 pg/mL (vs. 12.73 pg/mL in $CVID^+/CMV^+$ and 4.27 pg/mL in $CVID^+/CMV^-$).

DISCUSSION

The most striking clinical characteristics of our $CVID^+/CMV^+$ cohort were the high prevalence of monogenic CVID (66.7%, 4/6) and mortality rates (66.7%, 4/6 in 5 years). It is apparent from the case literature that treatment of CMV is a burdensome and often failed endeavor (9), but the potential impact of CMV disease on life expectancy in CVID has never been so starkly presented before.

Although just 7.6% of CVID patients managed by our unit had symptomatic CMV, they accounted for 66.7% (4/6) of deaths over the period of observation; therefore, the relative risk of death increased 26 times with the presence of CMV disease in this cohort. Only one death in the $CVID^+/CMV^+$ group was directly attributable to CMV. However, CMV's broad impacts on immune health (39) and the significant barriers to its treatment (as evidenced by one-third of our patients ceasing therapy due to cytopenias) suggest that CMV's indirect effects on morbidity and mortality in PID may be more significant than previously recognized.

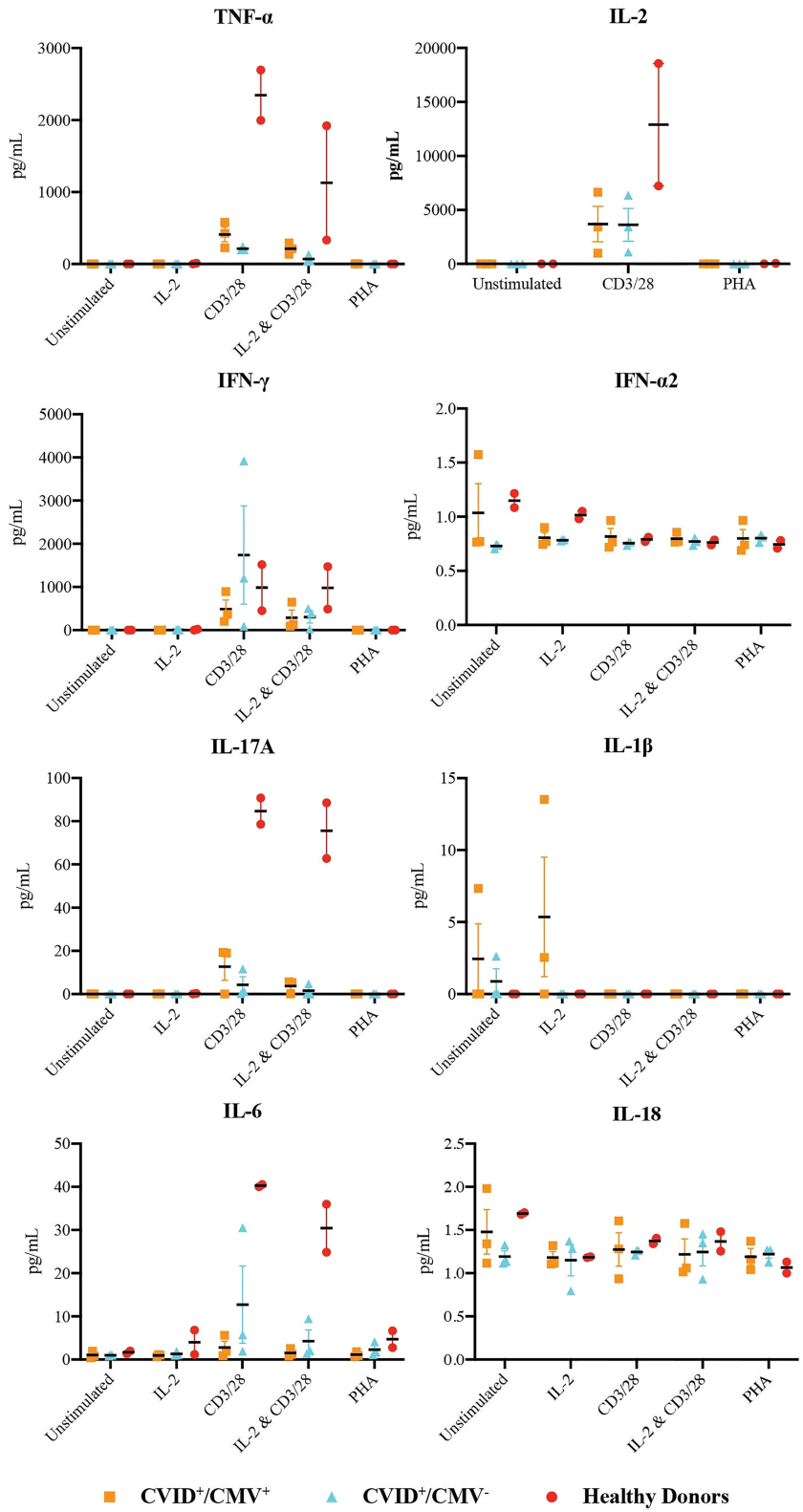


FIGURE 2 | Production of inflammatory cytokines by T-cells. Concentrations of TNF-α, IL-2, IFN-γ, IFN-α2, IL-17A, IL-1b, IL-6 and IL-1 in the supernatant of proliferating T-cells at 48 hours, under all stimulation conditions. Results presented as individual values, means and standard errors of the mean.

Given that monogenic causes of disease are generally identified in less than 20% of CVID (40), our finding of pathogenic variants in 4 of 6 patients is noteworthy. The frequency of mutations relating to the *NFKB* pathway in this group (3/6, 50%) is also of interest, given a recent report on overwhelming CMV infection in the context of a novel *NFKB2* mutation with reduced NK cell function (14). As the evidence base grows, it may be possible to propose further specific genetic abnormalities that contribute to CMV risk.

To our knowledge, this is the largest published case series of CMV disease in CVID. However, our finding of 6 cases of CMV disease in 79 individuals with CVID is comparable to the previously published finding of 3 affected patients in 32 individuals with CVID followed up for 335 patient-years (41).

'True' rates of CMV exposure, viraemia and disease are exceptionally difficult to evaluate in the context of PAD. Measurement of CMV-specific IgG is misleading in the setting of immunoglobulin replacement, use of serial molecular testing (CMV PCR) is a poorly established and costly assay, and tissue biopsy may carry high 'false negative' rates. For instance: the presence of CMV-specific $CD8^+$ T cells was reported in 55% of a UK CVID cohort (n=76), but genomic viral DNA was not detected in whole blood PCR (sensitivity 200 copies/mL of blood) in any of these patients, nor were CMV inclusion bodies demonstrated in three individuals strongly suspected to have CMV enteritis (11). On the other hand, another UK-based study found persistent peripheral CMV gene fragments in 4.9% (5/102) of their CVID patients, without any evidence of clinically relevant CMV disease in that cohort (42).

Rates of inflammatory disease in our patients with CMV disease (100%) were higher than the frequency reported in the pooled literature (77%) (9). This could be considered support for the hypothesis that an unrestrained T-cell response to CMV drives end-organ inflammation in CVID, even in the absence of detectable CMV viraemia or diagnostic histology. In a cohort of 42 patients with CVID, 73.8% (31/42) of individuals with inflammatory manifestations of CVID (hepatitis, splenomegaly, enteropathy or interstitial lung disease directly attributed to CVID) showed evidence of CMV exposure, vs. 25.8% (8/31) of individuals with an 'infections-only' phenotype (10).

It should be noted that lack of standardization in CMV testing is likely to result in a degree of ascertainment bias. Clinicians' threshold for CMV investigation is presumed to be lower where there is a history of significant iatrogenic immunosuppression, presenting a significant confounder to the apparent association between CMV and inflammatory disease. An alternative hypothesis to that of CMV directly causing inflammatory manifestations of CVID, is that individuals with inflammatory CVID are more likely to undergo genetic testing, more likely to require immunomodulatory treatment, and more likely to be investigated for CMV disease.

Our studies of T-cell proliferation and function were limited by the confounding factor of iatrogenic immunosuppression, small sample sizes, and lack of longitudinal data; in particular, samples pre-and post-onset of CMV disease would be valuable. Additionally, we were unable to analyse isolated $CD4^+$/$CD8^+$ T-cell proliferation or perform more extensive T-cell immunophenotyping for evaluation of LOCID, due to the low numbers of cells available for processing (in many cases due to the significant mortality rates in this cohort).

Most likely as a result of the small sample size, differences in the proportions of undivided to divided cells across the $CVID^+/CMV^+$, $CVID^+/CMV^-$ and healthy donor groups did not reach statistical significance for any of the time points and conditions studied. Similarly, there were no statistically significant differences between the three groups regarding cytokine concentrations in the supernatant produced by proliferating $CD3^+$ T cells at 48 hours. Nonetheless, several interesting trends emerged.

The finding of delayed $CD3^+$ proliferation to both CD3/28 activator and PHA has been demonstrated in previous case studies (23, 29), but is inconsistent with a report showing increased Ki-67 expression and decreased PD-1 expression (suggesting increased cell turnover and reduced cell exhaustion respectively) by $CD8^+$ CMV-specific T cells in patients with CVID previously exposed to CMV (10). Perhaps the functional profile of $CD8^+$ CMV-specific T cells is distinct from that of the wider $CD3^+$ T cell population. This may carry implications for inflammation, but is arguably less relevant to opportunistic infection risk than the broader assays undertaken in this study.

Kuntz et al.'s analysis of bulk $CD8^+$ T cells in 34 CVID patients demonstrated reduced numbers of $CCR7^+$ $CD8^+$ T cells and $PD\text{-}1^+$ $CD8^+$ T cells in individuals with CVID previously exposed to CMV, suggesting a more differentiated immunophenotype (29). This is in keeping with previous speculation that chronic CMV infection drives 'immuno-senescence', chiefly through clonal expansion of dysfunctional $CD8^+$ $CD28^-$ T cells that are anergic to stimulation with specific antigen (43).

Our analysis of supernatant cytokine concentrations is in contrast with previous studies reporting similar or higher levels of IFN-γ and TNF-α production in individuals with CVID compared with healthy donors. However, these experiments have typically been performed on virus-specific $CD8^+$ T cells (10). Furthermore, the spread of results in our supernatant experiments, particularly within the $CVID^+/CMV^-$ group, limit the reliability of this measure.

This single-centre retrospective cohort study suggests that CMV disease is a under-recognised manifestation of CVID and under-appreciated contributor to morbidity and mortality, particularly in the context of inflammatory disease and immunosuppression. An evidence base for CMV screening and treatment is sorely needed, given the significant challenges of treating CMV in the context of CVID, and CMV's potential implications for the possibility of curative treatment with HSCT. Assessment of CMV-specific T cell immunity, such as measurement of IFN-γ release by T cells in response to to CMV antigen (using either ELISPOT or QuantiFERON), are promising new assays for investigation of CMV-specific T cell deficits. However, these tests are not yet validated in PID (44–47).

Research in this area is hampered by the rarity and heterogeneity of PID. A targeted study of the CVID population presents an appealing launching pad for research to improve risk stratification, early identification and treatment of CMV disease:

a significant knowledge gap in contemporary PAD management, given the potential sequelae of CMV disease described in the literature and reiterated in our cohort study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research Ethics Committees of Melbourne Health (project reference number 2009.162) and WEHI (project reference number 10/02). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SC and JG wrote the manuscript. CS and VB conceptualised and designed the study and supervised the project. SC, MH, LH, AF, and MM performed the experiments and analysed the data. SC, JG, CJ, JC, JS, MY, JD, and CS provided the clinical datasets. All authors contributed to the article and approved the 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.2022.815193/full#supplementary-material

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Common Variable Immunodeficiency-Associated Cancers: The Role of Clinical Phenotypes, Immunological and Genetic Factors

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Objective: The aim of this study was to investigate the prevalence of cancer and associating clinical, immunological, and genetic factors in a German cohort of patients with common variable immunodeficiency (CVID).

Methods: In this retrospective monocenter cohort study, we estimated the standardized incidence ratio (SIR) for different forms of cancer diagnosed in CVID patients. Furthermore, we evaluated the likely association of infectious and non-infectious CVID-related phenotypes with the diagnosis of cancer by calculation of the odds ratio. The genetic background of CVID in patients with cancer was evaluated with sequential targeted next-generation sequencing (tNGS) and whole-exome sequencing (WES). Patients' family history and WES data were evaluated for genetic predisposition to cancer.

Results: A total of 27/219 patients (12.3%) were diagnosed with at least one type of cancer. Most common types of cancer were gastric cancer (SIR: 16.5), non-melanoma skin cancer (NMSC) (SIR: 12.7), and non-Hodgkin lymphoma (NHL) (SIR: 12.2). Immune dysregulation manifesting as arthritis, atrophic gastritis, or interstitial lung disease (ILD) was associated with the diagnosis of cancer. Furthermore, diagnosis of NMSC associated with the diagnosis of an alternative type of cancer. Studied immunological parameters did not display any significant difference between patients with cancer and those without. tNGS and/or WES yielded a definite or likely genetic diagnosis in 11.1% of CVID patients with cancer. Based on identified variants in cancer-associated genes, the types of diagnosed cancers, and family history data, 14.3% of studied patients may have a likely genetic susceptibility to cancer, falling under a known hereditary cancer syndrome.

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Conclusions: Gastric cancer, NMSC, and NHL are the most frequent CVID-associated types of cancer. Manifestations of immune dysregulation, such as arthritis and ILD, were identified as risk factors of malignancy in CVID, whereas studied immunological parameters or the identification of a monogenic form of CVID appears to have a limited role in the evaluation of cancer risk in CVID.

Keywords: CVID, cancer, CTLA-4, NF-κB1 (NF-kappaB1), cancer immune surveillance

INTRODUCTION

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency, comprising a heterogeneous group of disorders, which all associate with primary antibody production failure (1). Besides unusual infections, non-infectious manifestations including autoimmunity, autoinflammation, and polyclonal lymphoproliferation can precede the onset of clinically evident immunodeficiency and dominate the phenotype of CVID (2). According to a recent meta-analysis, the prevalence of malignancy in CVID was 8.6% (3). Most common types of cancer, which also display a relatively higher incidence in CVID patients, are lymphomas and gastric cancer (3–5). In contrast, there appears to be no association of CVID with other common types of cancer (6), such as lung or prostate cancer.

Next-generation sequencing (NGS) has considerably advanced our understanding of the genetic background of primary immunodeficiency disorders (PIDs) and led to the identification of an increasing number of monogenic forms of CVID (7). Furthermore, genetic studies provided new insights into the pathogenesis of PID-associated manifestations, such as autoimmunity and polyclonal lymphoproliferation (8). The same holds true for PID-associated cancers, where PID-associated genetic variants can either directly confer susceptibility to cancer or indirectly support carcinogenesis by causing genetic instability, persistent lymphoproliferation, and/or oncogenic infections (9, 10). Notable monogenic PIDs, whose genetic background directly predisposes to cancer, include the activated phosphoinositide 3-kinase δ syndrome (APDS), nuclear factor kappa B subunit 1 (NF-κB1) insufficiency, and Signal transducer and activator of transcription 3 (STAT3) gain-of-function syndrome (11–13). Evidence for this stems from the detection of somatic mutations in *PIK3CD*, *NFKB1*, and *STAT3* in human cancer genomes and the identification of their role in carcinogenesis (14–17).

The association of CVID with malignancy has not been integrated into clinical practice, and patients are not routinely offered screening for prevention and early diagnosis of cancer. The identification of the prevalence and relative incidence of cancer in CVID could raise awareness of malignancy as a major manifestation of CVID. Furthermore, the characterization of comorbidities, immunological abnormalities, or genetic factors associating with the diagnosis of cancer in CVID could improve our understanding of carcinogenesis in CVID and eventually contribute to the development of effective cancer screening. This monocentric retrospective study was designed to evaluate the clinical phenotypes, the immunological profiles, and the genetic factors associating with the diagnosis of cancer in CVID.

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated protein 4; CVID, common variable immunodeficiency; DSR, double-strand repair; ILD, interstitial lung disease; NF-κB1, nuclear factor kappa B subunit 1; NHL, non-Hodgkin lymphoma; NMSC, non-melanoma skin cancer; SIR, standardized incidence ratio; tNGS, targeted next-generation sequencing.

MATERIALS AND METHODS

Study Cohort

This study included a total of 219 patients with CVID visiting the immunology outpatient clinics of the Department of Rheumatology and Immunology of the Hannover Medical School. Data were collected from 2015 to 2021. Following comment has been added: Diagnosis of CVID was based on the current European Society for Immunodeficiencies (ESID) diagnostic criteria [available at http://esid.org/Working-Parties/Registry/Diagnosis-criteria (18)] or the original ESID/Pan-American Group for Immunodeficiency (PAGID) (1999) criteria (available at https://esid.org/Education/Common-Variable-Immunodeficiency-CVI-diagnosis-criteria). Among those originally diagnosed on the basis of the ESID/PAGID criteria, this study included only patients with reduced immunoglobulin A (IgA) levels, poor antibody response to vaccines, or absent isohemagglutinins or low class-switched memory B cells and $CD4^+$ T-cell counts >200/μl. Immunological and clinical data were obtained from patients' medical files. Immunological data included serum immunoglobulin levels and counts of major lymphocyte subsets at diagnosis of CVID. Documented clinical data included clinical history of infections, bronchiectasis [computed tomography (CT)-confirmed], autoimmune cytopenias, such as autoimmune hemolytic anemia (AIHA), idiopathic thrombocytopenic purpura (ITP), organ-specific autoimmunity [including vitiligo, psoriasis, insulin-dependent diabetes mellitus (IDDM), thyroidopathies, atrophic gastritis, and arthritis], granulomatous disease, enteropathy, and malignancies. CVID-associated interstitial lung disease (ILD) was diagnosed based on typical CT scan findings in the absence of evidence for an infectious or alternative cause. Splenomegaly was defined as spleen enlargement ≥11 cm on palpation or ultrasound, including previous splenectomy of an enlarged spleen. Lymphadenopathy was detected on palpation, ultrasound, CT, or magnetic resonance scan. Granulomatous disease was defined as at least one biopsy-proven unexplained granuloma, excluding Crohn's disease-associated granulomas. Enteropathy included all cases of biopsy-proven non-infectious inflammatory bowel disease

(ulcerative colitis and Crohn's disease) and intestinal hyperlymphocytosis (lymphocytic infiltration of the interepithelial mucous, the lamina propria, and/or the submucosa). Malignancies included hematologic and all other forms of cancer. Diagnosis and staging of cancer were made according to relevant contemporary German Cancer Society guidelines (available at https://www.leitlinienprogramm-onkologie.de/leitlinien/). Briefly, colon cancer and gastric cancer were diagnosed with gastrointestinal endoscopy coupled with biopsies. Breast cancer was diagnosed with standard imaging techniques after histologic confirmation. Lymphomas were diagnosed on the basis of imaging, including CT, MRI, and PET, as well as histological examination of tissue sections, lymph nodes, and/or bone marrow biopsies. The diagnosis of non-melanoma skin cancer (NMSC) was made clinically and confirmed histologically after tumor excision. Family history of all 27 patients diagnosed with cancer has been assessed for a potential hereditary cancer syndrome based on the European Society for Medical Oncology (ESMO) guidelines for hereditary cancer syndromes (https://www.esmo.org/guidelines/hereditary-syndromes). In particular, each patient's family history has been retrospectively evaluated based on patients' medical files for following red flags of hereditary cancer: 1) more than two affected close relatives (parents, children, siblings, grandparents, aunts/uncles, and first cousins) from the same family side with same or related cancers (i.e., two or more of the following cancers: breast, ovarian, prostate, pancreatic; two or more of the following cancers: colorectal, endometrial, ovarian, gastric, pancreatic; two or more of the following cancers: breast, thyroid, endometrial, colorectal, melanoma, kidney); 2) diagnosis of cancer before the age of 50 years; 3) rare forms of cancer (such as ovarian cancer, sarcomas, adrenocortical carcinoma, choroid plexus carcinoma); 4) Ashkenazi Jewish ancestry; and 5) consanguinity. In case of 20/27 studied patients, we were additionally able to take a structured family history during the current study, focusing on family history of cancer and all the abovementioned red flags of hereditary cancer.

All patients signed an informed consent form. This study was approved by the ethics review board of the Hannover Medical School Ethics Committee (ethics vote number: 5582; 8875_BO_K_2020).

Next-Generation Sequencing

Blood samples were collected in the immunology outpatient clinics of the Department of Rheumatology and Immunology of the Hannover University School. Genomic DNA was extracted by QIAamp DNA Blood Midi Kit (Qiagen) and quantified by Qubit dsDNA BR Assay Kit (Thermo Fisher). All 27 patients diagnosed with cancer were sequenced by means of targeted next-generation sequencing (tNGS), which was performed as described previously (19). Briefly, a customized panel of genes associated with PIDs (19) was created with the help of Agilent's web-based SureDesign application. DNA target enrichment was performed using Agilent's HaloPlex Target Enrichment System for Illumina sequencing following the manufacturer's instructions (Agilent's user manual). Sequencing was performed on an Illumina MiSeq system using an Illumina v2 reagent kit following the manufacturer's protocol. The FastQ files were aligned to the human reference genome (UCSC hg19, *GRCh37*) and analyzed using Agilent's SureCall software as described previously (19) Whole-exome sequencing (WES) was performed on genomic DNA samples from 21/27 patients diagnosed with cancer as described previously (20). Briefly, the concentration and quality of the purified genomic DNA (gDNA) were determined with an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The DNA sequencing library consisted of 100 ng fragmented gDNA and was generated with Agilent SureSelectXT Reagent Kits v5 UTR (70 Mb) according to the manufacturer's protocols (Illumina, San Diego, CA, USA). Libraries were sequenced on an Illumina HiSeq2500 platform using TruSeq SBS Kit v3-HS (200 cycles, paired end run) with an average of 12.5 × 106 reads per single exome (mean coverage: 50×). The GATK-Pipeline (GenomeAnalysisTK-1.7) was applied for read quality trimming, read alignment to reference (UCSC hg19, GRCh37), and quality trimmed variant calling. Variant annotation was performed using Gsvar software. Patient exomes were filtered for mutations in 490 genes associated with PIDs as well as 125 cancer-associated genes (**Supplementary Table 1**). Genetic variants in PID and cancer-associated genes were excluded if the allele frequencies in the general population were >1% in the Exome Aggregation Consortium database (ExAC) or the Genome Aggregation Database (gnomAD). Besides allele frequency, identified variants were selected according to the following criteria: variant annotation and potential functional effect using databases of variants [e.g., dbSNP, 1000 Genomes Project, Exome Aggregation Consortium (ExAC), gnomAD] and disease-causing variants [Human Gene Mutation Database (HGMD), Online Mendelian Inheritance in Man (OMIM)]. Furthermore, we kept nonsense variants, variants affecting splice site, frameshift, in-frame indels, start or stop codon changes, as well as missense variants that were predicted deleterious by having a combined annotation-dependent depletion (CADD) score ≥20 and a mutation significance cutoff (MSC) score below the CADD score (21, 22). Targeted sequencing findings are publicly available under following links: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA750325/PRJNA750325.

Expression of Cytotoxic T-Lymphocyte-Associated Protein 4

Whole blood was collected in ethylene diamine tetraacetic acid (EDTA) tubes, and peripheral blood mononuclear cell (PBMC) preparations have been performed as described previously (23). PBMCs were cryopreserved in freezing medium (heat-inactivated fetal calf serum (FCS), PAN-Biotech; 10% v/v dimethyl sulfoxide, Sigma) until use. Upon cell thawing, PBMCs were rested overnight in complete RPMI medium (at 37°C, 5% CO_2) for recovery and collected in the morning for further processing. After washing once in fluorescence-activated cell sorting (FACS) buffer [phosphate buffered saline (PBS), 5% v/v FCS], the cells were stained with antibodies in the presence of Octagam 10% (Octapharma). PBMCs were stained with CD4-BV421 (clone RPA-T4, BD Biosciences), CD25-BV510 (clone 2A3, BD Biosciences-OptiBuild), and CD127-PE (clone A019D5, BioLegend) antibodies and Fixable Viability Dye eFluor780

(Thermo Fisher). Regulatory T cells (Tregs) (live $CD4^+$ $CD25^{hi}$ $CD127^{lo}$) were sorted under aseptic condition in BD FACSAria Fusion cell sorter (Becton-Dickinson). Cell sorting typically yielded high enrichment (>90%) of Tregs. Sorted Tregs were stimulated with anti-CD3/CD28 beads (Dynabeads, Thermo Fisher) for 16 h. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression was assessed in Cyto-Fast Fix/Perm (BioLegend)-permeabilized cells using a CTLA-4-APC antibody (BioLegend).

Statistical Analysis

For statistical calculation, we used GraphPad prism 9 (GraphPad, La Jolla, USA). Descriptive statistics are reported as median and interquartile range (IQR) in case of continuous variables and as counts and percentages for dichotomous variables. Categorical variables were compared by the Fisher's exact test. Differences between patients with and without cancer were evaluated with the Mann–Whitney test. Comparison of more than two groups was performed with the Kruskal–Wallis test. To correct for multiple testing, p values were adjusted for Benjamini–Hochberg false discovery rate (FDR). The p values were considered significant if they were lower than a threshold selected to control an FDR of 10%. To calculate standardized incidence ratios (SIRs), all patients were stratified by age and gender. Data on the reference population were derived from the 12th edition of the "Cancer in Germany" report by the Robert Koch Institute (23). The expected number of cases for each type of cancer in the studied patient cohort was calculated based on the age- and gender-specific incidence rate provided in the aforementioned report. SIR was calculated by dividing the actual number of cases in the studied patient cohort by the one in the reference population.

RESULTS

Clinical Characterization of Common Variable Immunodeficiency Patients

Patients' demographic data and characteristics are summarized in **Table 1**. Most patients had sporadic immunodeficiency. Besides recurrent infections, most patients had a history of at least one non-infectious CVID-associated manifestation (157/219, 71.7%). Those included benign lymphoproliferation, manifesting as lymphadenopathy and/or splenomegaly (107/219, 48.9%), autoimmune disease (82/219, 37.4%), or atopic disease (37/219, 16.9%). A total of 27 patients (12.3%) were diagnosed with at least one form of cancer. Their clinical characteristics, including cancer type and stage, are summarized in **Supplementary Table 2**. Median age of first diagnosis of cancer was 45 years (IQR: 37–58 years). In most cases, the first diagnosis of cancer followed the diagnosis of CVID (20/27, 74.1%). The median time from diagnosis of CVID to diagnosis of cancer was 4 years (IQR: 1–13 years). Furthermore, 7/219 patients (3.2%) were diagnosed with more than one form of cancer. Diagnosis of cancer was equally common in sporadic and familial cases of CVID (3/27 vs. 17/192; p = 0.72). At the time of data analysis, 5/27 patients were deceased, and one was lost to follow-up (**Supplementary Table 2**).

TABLE 1 | Characteristics of studied patients (*N* = 219).

Median age at diagnosis of CVID[1], years (IQR)	33 (21–45)
Male sex, no. (%)	91 (41.6)
Familial cases, no. (%)	20/219 (9.1)
History of parental consanguinity, no. (%)	2/219 (0.9)
Recurrent pneumonias[2], no. (%)	148 (67.6)
Bronchiectasis, no. (%)	51 (23.3)
Recurrent gastrointestinal infections[2], no. (%)	46 (21)
"Infections only" disease, no. (%)	62 (28.3)
Benign lymphoproliferation, no. (%)	103 (47)
Splenomegaly, no. (%)	22 (10)
Enteropathy, no. (%)	27 (12.3)
ILD, no. (%)	22 (10)
ITP, no. (%)	32 (14.6)
AIHA, no. (%)	13 (5.9)
Psoriasis, no. (%)	10 (4.6)
Vitiligo, no. (%)	8 (3.7)
Thyroidopathy, no. (%)	14 (6.4)
Atrophic gastritis, no. (%)	6 (2.7)
Arthritis, no. (%)	16 (7.3)
Atopic disease[3], no. (%)	37 (16.9)
Granulomatous disease, no. (%)	25 (11.4)
Cancer, no. (%)	27 (12.3)
Immunoglobulin replacement, no. (%)	205 (93.6)
Immunosuppressive regimens, no. (%)	74 (33.8)

AIHA, autoimmune hemolytic anemia; CVID, common variable immunodeficiency; ILD, interstitial lung disease; IQR, interquartile range; ITP, immune thrombocytopenic purpura; no., number.

[1]*Analysis based on 202/219 studied patients with known year of diagnosis.*

[2]*At least two documented pneumonias/gastrointestinal infections.*

[3]*Atopic dermatitis and/or allergic rhinitis and/or asthma.*

Prevalence and SIR for different types of cancer are presented in **Table 2**. Gastric cancer followed by NMSC and non-Hodgkin lymphoma (NHL) appeared to be considerably more common in CVID, displaying the highest SIR values. Besides the higher prevalence of those three forms of cancer, all of them appear to be diagnosed considerably earlier in CVID than in the general population (**Supplementary Table 3**) (24). Interestingly, gastric cancer was diagnosed at an early stage and at a considerably younger age (median: 36.5 years, IQR: 35–69) than in the general population (24). Regarding NHL, similar to the general population (25), most CVID patients (5/6) developed B-cell lymphomas. Furthermore, all cases of breast cancer were early-stage hormone receptor (HR) positive and human epidermal growth factor receptor (HER-2) negative, which is the most prevalent breast cancer in the general population (26).

TABLE 2 | Prevalence and SIR for different types of cancer in a cohort of 219 CVID patients.

Cancer	Prevalence N (%)	SIR (95% CI)
Breast cancer	6 (2.74)	1.87 (0.6–4.36)
Lung cancer	1 (0.46)	0.69 (0.9–3.82)
NHL	6 (2.74)	12.2 (4.46–26.57)
Gastric cancer	6 (2.74)	16.54 (6.04–36.01)
Colorectal cancer	4 (1.83)	2.8 (0.75–7.18)
NMSC	8 (3.65)	12.74 (5.1–26.27)

CVID, common variable immunodeficiency; NMSC, non-melanoma skin cancer; NHL, non-Hodgkin lymphoma; SIR, standardized incidence ratio.

Common Variable Immunodeficiency Manifestations Associating With Cancer

Considering all 219 patients, arthritis, atrophic gastritis, and ILD were the CVID-associated manifestations, which were significantly more common among patients with cancer (**Table 3**). Furthermore, NMSC was diagnosed in 5/7 with more than one type of cancer. Most of those 5 patients (4/5) had received no radiation therapy or chemotherapy prior to diagnosis of NMSC, which would result in increased risk for NMSC (27). NMSC, and in particular basal cell carcinoma, significantly associated with the diagnosis of an additional form of cancer [5/24 vs. 3/195; $p < 0.005$; odds ratio (OR): 16.84, IQR: 3.73–76.04]. In 3 out of 5 cases with a diagnosis of more than one form of cancer, including NMSC, diagnosis of NMSC preceded the diagnosis of an additional form of cancer. A subgroup analysis was performed for the relatively more common forms of cancer, i.e., NMSC, NHL, gastric cancer, and breast cancer. As reported in previous studies (4), CVID patients with gastric cancer were more commonly diagnosed with atrophic gastritis ($p = 0.009$; OR = 26.11, IQR: 3.67–186.4) and had a history of recurrent gastrointestinal infections ($p = 0.0187$; OR = 8.14, IQR: 1.44–45.98) or infection with *Helicobacter pylori* ($p = 0.0256$; OR = 12.81, IQR: 2.04–80.59). For the rest of the diagnosed forms of cancer, we identified no significant association with any of the studied CVID manifestations. Also treatment with immunoglobulin replacement or immunosuppressive agents did not associate with the diagnosis of cancer (**Table 4**).

The Role of Immunological and Genetic Parameters

Among patients' immunological parameters, levels of main classes of immunoglobulins and counts of $CD19^+$ B cells, natural killer (NK) cells, and $CD4^+$ and $CD8^+$ T cells at diagnosis of CVID were

TABLE 3 | Association of infectious and non-infectious manifestations of CVID with cancer.

	Variable	At least one cancer (*N* = 27)	No cancer (*N* = 192)	OR (95% CI)	p^+	*q*
immune dysregulation	AIHA	2	11	1.32 (0.28–6.29)	0.6654 (ns)	0.8555
	ITP	5	27	1.39 (0.48–3.98)	0.5611 (ns)	0.7769
	Arthritis	5	10	4.14 (1.3–13.2)	0.0246 (ns)	0.1476
	Atopic disease	6	31	1.48 (0.55–3.98)	0.4171 (ns)	0.7508
	Atrophic gastritis	4	2	16.52 (2.87–95.28)	0.0024 (**)	0.0432
	Enteropathy	5	22	1.76 (0.6–5.11)	0.3442 (ns)	0.7508
	Granulomatous disease	5	20	1.96 (0.67–5.73)	0.2064 (ns)	0.6192
	ILD	7	14	4.45 (1.61–12.32)	0.0070 (**)	0.0630
	Lymphadenopathy	15	88	1.48 (0.66–3.23)	0.4119 (ns)	0.7508
	Psoriasis	1	9	0.78 (0.1–6.43)	1.000 (ns)	1.0000
	Splenomegaly	4	18	1.68 (0.52–5.4)	0.4895 (ns)	0.7769
	Thyroidopathy	2	13	1.1 (0.23–5.17)	1.000 (ns)	1.0000
infectious manifestations	Bronchiectasis	5	42	0.81 (0.29–2.27)	0.8064 (ns)	0.9255
	Infections only disease	7	57	0.83 (0.33–2.1)	0.8227 (ns)	0.9255
	H. pylori	3	7	3.3 (0.8–13.64)	0.1111 (ns)	0.5000
	Gastrointestinal infections[1]	8	38	1.71 (0.69–4.19)	0.3107 (ns)	0.7508
	Pneumonias	15	132	0.57 (0.25–1.29)	0.1922 (ns)	0.6192
	Shingles	2	27	0.49 (0.11–2.19)	0.5440 (ns)	0.7769

AIHA, autoimmune hemolytic anemia; CI, confidence interval; CID, combined immunodeficiency; CVID, common variable immunodeficiency; ILD, interstitial lung disease; ITP, immune thrombocytopenic purpura; ns, not significant; OR, odds ratio; PID, primary immunodeficiency disorder; RR, risk ratio; SPAD, specific antibody deficiency.

$^+p < 0.05$; $^{**}p < 0.01$.

[1]Other than with H. pylori.

TABLE 4 | Association of patients' treatment with cancer.

Variable	At least one cancer (*N* = 27)[1]	No cancer (*N* = 192)	OR (95% CI)	p
Immunoglobulin replacement	23	180	0.83 (0.18–3.95)	0.6852 (ns)
Immunosuppressive treatment	8	66	0.8 (0.33–1.93)	0.67 (ns)
-Systemic glucocorticoidmonotherapy	3	27	0.76 (0.22–2.71)	1 (ns)
-csDMARD based regimen	2	27	0.49 (0.11–2.19)	0.544 (ns)
–AZA	1	9	0.78 (0.1–6.43)	1 (ns)
–MTX	1	10	0.7 (0.09–5.67)	1 (ns)
-bDMARD-based regimen	2	11	1.66 (0.35–1.66)	0.6284 (ns)
–RTX	2	6	2.48 (0.47–12.97)	0.2573 (ns)
–TNFi	0	3	0.98 (0.05–19.59)	1 (ns)

AZA, azathioprine; bDMARD, biological disease-modifying antirheumatic drug; csDMARD, conventional synthetic disease-modifying antirheumatic drug; MTX, methotrexate; OR, odds ratio; RTX, rituximab; TNFi, tumor necrosis factor inhibitor.

[1]For patients with cancer, we considered treatment prior to first diagnosis of cancer.

available for most patients. We identified no significant differences in the levels of immunoglobulins (**Figure 1**) or the studied lymphocyte or B-cell subsets (**Figures 2**, **3**) between patients with cancer and those without. Furthermore, to identify the genetic background of immunodeficiency, all 27 patients with cancer were initially subjected to genetic testing by means of tNGS. A male patient (patient 3), who harbored a monoallelic variant in *NFKB1* (c.904dupT; p. S302Ffs*7), has been already reported by Schröder et al. (28). Furthermore, tNGS detected a novel *CTLA-4* variant (c.118G>A; p. V40M) in a female with human papillomavirus (HPV)-associated cervical cancer (patient 10). Pathogenicity of this variant was suggested by identifying reduced baseline and activation-induced CTLA-4 expression by patient's regulatory T cells with flow cytometry (**Figure 4**). Considering allele frequency as well as the combined annotation dependent depletion (CADD) and sorting intolerant from tolerant (SIFT) values of each identified variant, we found 3 predicted pathogenic monoallelic variants in *TNFRSF13B* in two studied patients (patient 1 and patient 25; **Supplementary Table 4**). Variants in *TNFRSF13B*, which encodes the transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), are commonly detected in patients with CVID and are considered disease-predisposing rather than disease-causing (29). As the employed tNGS panel included a limited number of CVID and PID-associated genes, we tested 20/27 patients with WES, for whom DNA was available, searching for variants in all known PID-associated genes. In addition to the abovementioned PID-associated variants, WES detected two *TTC7A* variants in a patient (patient 7), who besides cancer displayed recurrent respiratory tract infections and no further CVID-associated manifestations. Biallelic *TTC7A* mutations have been shown to cause combined immunodeficiency associating with early-onset inflammatory bowel disease (IBD) and atresia (30, 31). Despite the fact that the allelic phase of detected *TTC7A* variants has not been tested, lack of any clinical or endoscopic evidence of IBD or structural intestinal defect in this patient makes the diagnosis of TTC7A defect unlikely. All predicted pathogenic variants, identified through tNGS or WES, including the *TNFRSF13B* ones, are listed in **Supplementary Table 4**. Overall, only 2/27 (7.4%) had a definitive genetic diagnosis.

Cancer Genetic Risk Assessment

To evaluate the possibility of an underlying hereditary cancer predisposition syndrome, we retrospectively evaluated family history documentation, which was available in case of 25/27 studied patients with cancer. In addition, we were able to take family history, focusing on cancer and red flags of hereditary cancer in 20/27, who were not lost to follow-up. Family history data of 25/27 patients with cancer are summarized in **Supplementary Table 5**. Among the studied red flags of hereditary cancer, no patient had a history of consanguinity or an Ashkenazi Jewish ancestry. Elicited family history red flags of hereditary cancer included more often multiple related cancers on the same side of the family or relatively early onset of cancer in patients' close relatives. Overall, at least one red flag was present in 11/25 (44%) [or 8/20 (40%) in case family history was taken during the study].

Next, we analyzed WES data, which were available for 21/27 CVID patients with cancer, focusing on genes associating with hereditary cancer syndromes (32–34). We detected 18, all monoallelic cancer-associated variants in 12/21 studied subjects, 5 of whom had a family history with at least one hereditary cancer red flag (**Supplementary Table 6**). Most common were missense variants (13/18). With respect to the mechanism of carcinogenesis, most identified variants were identified in genes involved in DNA repair (10/18) and especially double-strand DNA repair (DSR) (6/18; see **Supplementary Table 6**). Interestingly, 3 out of 7 tested patients diagnosed with basal cell carcinoma (patient 1, patient 6, and patient 12) harbored at least one variant in genes involved in DNA repair, which might be relevant for the development of NMSC (35). WES detected no known pathogenic variants, which would lead to the diagnosis of a hereditary cancer syndrome. Genetic variants of unclear significance were however detected in genes such as *CDH1*, *DICER1*, *MEN1*, *RET*, *CREBBP*, *RAD51C*, or *SOS1*, which are linked to autosomal dominant predisposition to

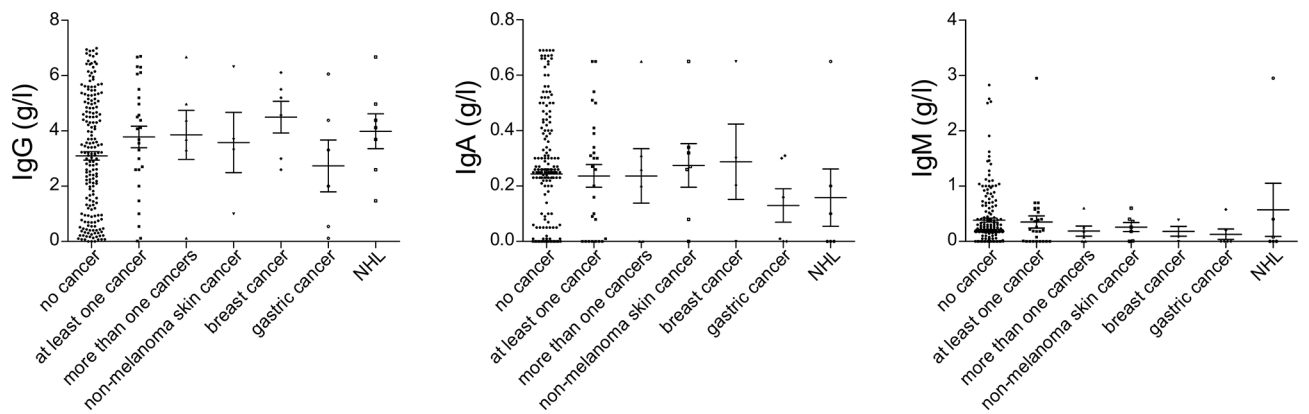

FIGURE 1 | Serum immunoglobulin levels at diagnosis of common variable immunodeficiency (CVID), prior to the introduction of immunoglobulin replacement, in patients with no cancer (*N* = 192), patients with at least one cancer (*N* = 27), more than one type of cancer (*N* = 6), non-melanoma skin cancer (*N* = 7), breast cancer (*N* = 7), gastric cancer (*N* = 6), and non-Hodgkin lymphoma (NHL, *N* = 6). No significant differences could be detected.

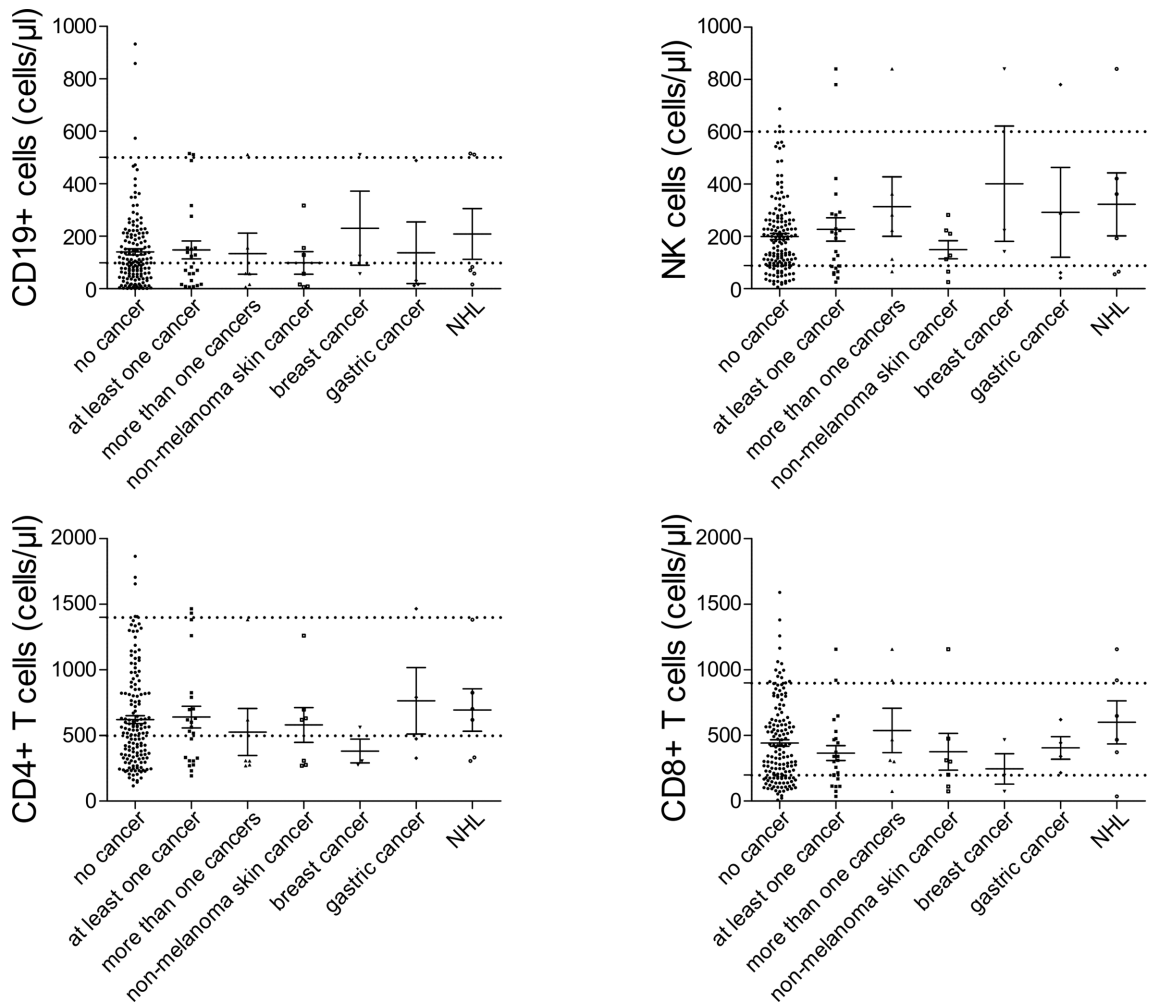

FIGURE 2 | Peripheral lymphocyte subset counts at diagnosis of common variable immunodeficiency (CVID) in patients with no cancer (*N* = 192), patients with at least one cancer (*N* = 27), more than one type of cancer (*N* = 6), non-melanoma skin cancer (*N* = 7), breast cancer (*N* = 7), gastric cancer (*N* = 6), and non-Hodgkin lymphoma (NHL, *N* = 6). No significant differences could be detected.

cancer (36–43). Given the gene-specific mode of inheritance of cancer susceptibility, the presence of a suggestive family history and the types of diagnosed cancers, 3/21 (14%) WES-tested patients (i.e., patient 1, patient 2, and patient 6; **Supplementary Table 6**) may have a genetic susceptibility to cancer, falling under a known hereditary cancer syndrome. Identified monoallelic variants in genes linked to autosomal recessive predisposition to cancer such as the ones in *RAD50*, *FANCC*, and *FANCM* might also be relevant for cancer risk (44–46).

DISCUSSION

Previous studies reported a variable prevalence of cancer in patients with CVID and identified lymphomas and gastric cancer as the most common CVID-associated malignancies (9, 10, 47). The relatively high prevalence of those two cancer types in the studied cohort is in line with previously presented case series of CVID. However, the identification of NMSC, and in particular basal cell carcinoma, as the most prevalent CVID-associated cancer deviates from most previous studies (9). This could be explained through the underrecording of cases in previous reports and/or the rising incidence rates of NMSC in the last decades (48). Furthermore, studies on CVID-associated cancers can be confounded by variations between patient cohorts and especially the regional variability in the prevalence of the diverse types of cancer (49, 50). Evaluation of relative occurrence of different forms of cancer in CVID through the calculation of its prevalence can be biased, given the differences in the demographic parameters between studied cohorts and the general population. The estimation of SIRs in the present study

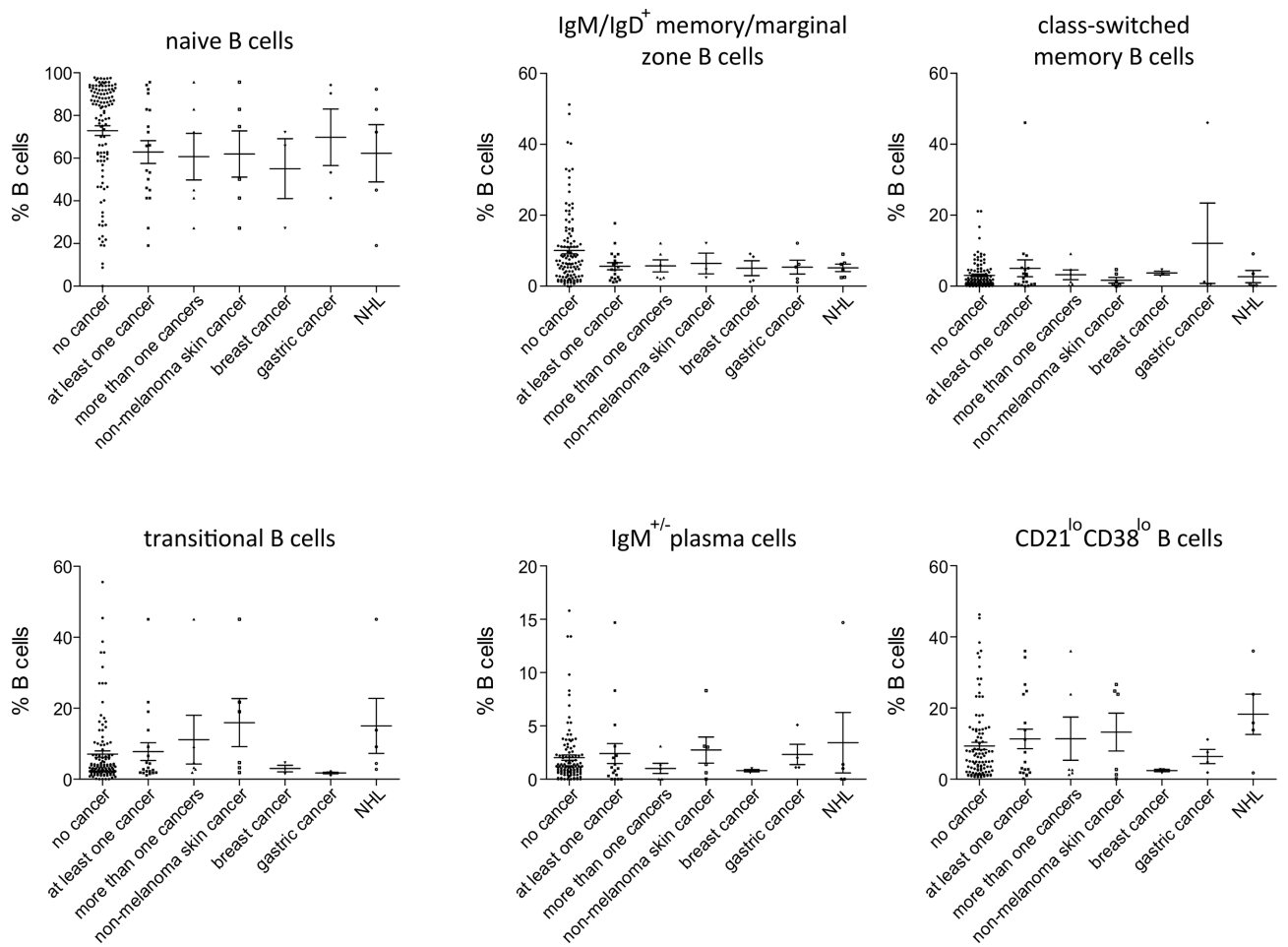

FIGURE 3 | Peripheral B-cell subset in patients with no cancer (*N* = 116), patients with at least one cancer (*N* = 19), more than one type of cancer (*N* = 6), non-melanoma skin cancer (*N* = 6), breast cancer (*N* = 3), gastric cancer (*N* = 4) and non-Hodgkin lymphoma (NHL, *N*=5). No significant differences could be detected.

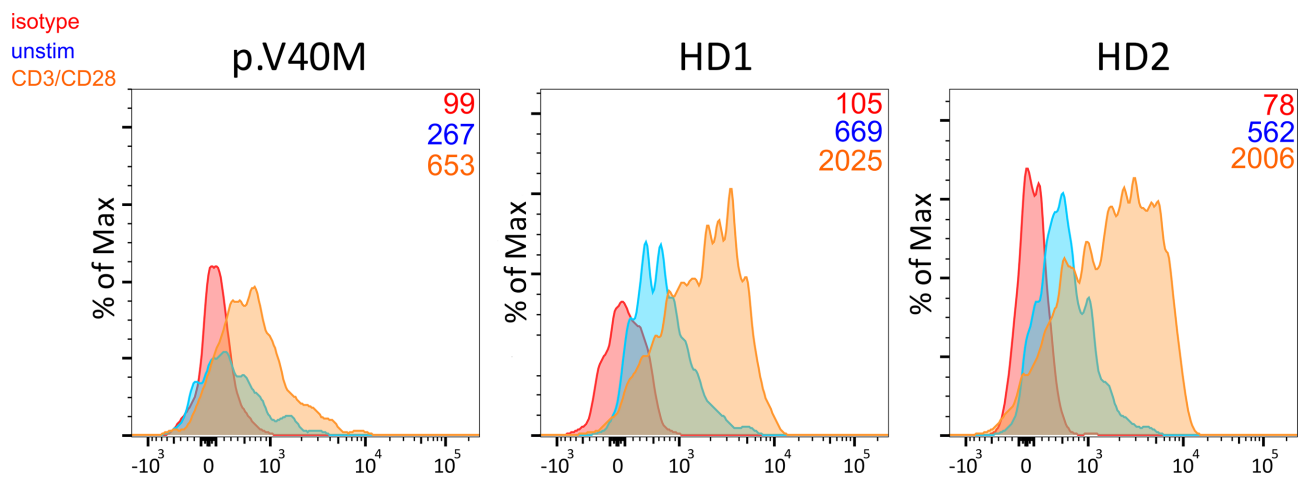

FIGURE 4 | Decreased baseline and CD3/CD28 activation-induced CTLA-4 expression in $CD4^+$ $CD25^{hi}$ $CD127^{lo}$ Treg from a patient harboring the c.118G>A (p.V40M) variant in CTLA-4 and two healthy blood donors. Median fluorescence intensity of CTLA-4 is shown (numbers) for each studied subject.

highlights the considerably higher occurrence of gastric cancer, NMSC, and NHL in CVID patients as compared to the general population. Besides its association with particular types of cancer, CVID may affect their outcome (9), which needs to be evaluated in studies following up larger numbers of CVID patients with the same forms of cancer.

Despite the fact that the presence of profound T-cell defects precludes the diagnosis of CVID and rather suggests the diagnosis of a combined immunodeficiency, CVID patients can display variable milder cellular defects (18). The degree of cellular immunodeficiency in CVID could associate with the diagnosis of cancer, which would be compatible with the concept of the immune surveillance of tumors (51). In fact, according to a previous report on a cohort of 801 patients with primary hypogammaglobulinemia, including CVID, patients with cancer displayed lower $CD8^+$ T-cell counts and patients with non-hematological malignancies had in addition significantly lower B cells (52). In a recent meta-analysis of 48 studies with more than 8,000 CVID patients, those with malignancies tended to display lower percentages of $CD8^+$ T cells (3). However, $CD8^+$ T-cell percentages were not significantly different, and percentages of the rest of the studied lymphocyte subsets were similar between CVID patients with cancer and those without. Here, evaluation of patients' immunological parameters at diagnosis of CVID, including B cells, NK cells, and $CD8^+$ T-cell counts, revealed no significant differences between patients with cancer and those without. Lack of significant changes in NK or $CD8^+$ T cells seemingly contradicts the concept of tumor immune surveillance. Nonetheless, T-cell or NK cell dysfunction has been reported in patients with CVID and may be relevant for CVID-associated carcinogenesis (53–55). Functional characterization of T cells or NK cells is not routinely performed in CVID, and their role as a predictor of carcinogenesis in CVID needs to be evaluated.

The increasing availability of NGS technologies has led to the increasing identification of the genetic background of CVID and to a growing proportion of monogenic disorders, which manifest as CVID. According to recent reports, the proportion of monogenic forms has increased, exceeding 20% of cases (56–58). In the present work, most sequenced patients were sporadic cases. This together with the fact that not all studied patients were subjected to WES may account for the relatively low percentage of patients detected with pathogenic variants. However, in a recent study evaluating the genetic background of immunodeficiency through whole-genome sequencing (WGS) in a large patient cohort mainly consisting of sporadic PID, only approximately 10% of tested patients were identified to harbor pathogenic or likely pathogenic genetic variants (58). Germline mutations in *CTLA4* or *NFKB1* can cause CVID and at the same time associate with an increased risk of malignancy (12, 56). A previous study addressing the genetic cause of CVID in 10 patients with lymphoma revealed a heterogeneous genetic background, including a patient with CTLA4-insufficiency and one with APDS as a consequence of a monoallelic gain-of-function variant in *PIK3CD* (5). Identification of two cancer patients with monogenic CVID in this study, one with CTLA-4 insufficiency and another with NF-κB1 defect, comes in line with previous reports, suggesting the higher risk of malignancy in those monogenic disorders. Similar to the study by Kralickova et al. (5), the prevalence of monogenic disorders among patients with CVID and cancer was relatively low. Despite that, genetic diagnosis of particular disorders, which associate with a higher cancer risk, such as NF-κB1 defect or CTLA-4 insufficiency, should urge treating physicians to consider regular cancer screening.

Besides the identification of the genetic basis of PID and the diagnosis of monogenic inborn errors of immunity, NGS technologies, and in particular higher throughput ones (i.e., WES or WGS), can be useful in identifying cancer risk conferring genetic variants in CVID. The latter may aid in identifying CVID patients at higher risk for malignancies and might consequently lead to intensified cancer screening for this patient subgroup. In the present study, a third of tested patients harbored a predicted pathogenic variant that may lead to hereditary susceptibility to cancer, and less than 25% had in addition a family history, which was suggestive of a hereditary cancer syndrome. With respect to the identified variants, the majority have been identified in genes involved in DNA repair, especially DSR. The latter is in accordance with the previously reported high frequency of genetic variation in DNA repair genes in CVID (57, 58). A genetic background affecting DNA repair would be in line with studies demonstrating chromosomal radiosensitivity (59, 60) and DSR defects in CVID (58) as well as with the identified higher prevalence of NMSC, whose key risk factor is the ultraviolet radiation through its DNA damage-inducing potential (61).

Our study has several limitations. For the calculation of SIR, despite gender and age stratification, the total German population was employed as reference population. Detailed geographical matching of the patients was not possible due to the lack of data from different regions of Germany. However, lack of substantial differences in the incidence of CVID-associated malignancies across different German federal states suggests that geographical matching would have no major influence on SIR values for most forms of cancer. The limited number of patients diagnosed with malignancies and relatively common cancer forms such gastric cancer and lymphomas hampers the identification of immunological risk conferring factors as well as the evaluation of the outcome of particular forms of cancer in CVID. Finally, evaluation of pathogenicity of variations in cancer-associated genes was hampered by the lack of functional testing to identify their likely role in carcinogenesis and sequencing data from family members, which would enable segregation analysis.

Despite the identification of cancer as a main non-infectious manifestation of CVID (9), there is no consensus guideline for cancer screening. Cancer screening could be especially relevant for the outcome of gastric, colorectal, and breast cancer (62), which are common forms of cancer in CVID. In the present study, manifestations of immune dysregulation associated with the diagnosis of cancer in CVID. Furthermore, basal cell carcinoma, which in most cases can be cured with surgical excision (26), associated with the diagnosis of an alternative type of cancer in CVID. Independently of the diagnosis of CVID or an alternative PID, germline mutations have been shown to play an important role

in case of early-onset and/or recurrent basal cell carcinomas and to associate with increased risk for other forms of cancer (63, 64). The identification of such cancer-associated manifestations together with the integration of tools/questionnaires (65) and/or relevant information from high-throughput genetic data (i.e., WES or WGS), which are becoming all the more available in daily clinical practice of PID, could aid the development of cost-effective screening programs, which may improve patients' outcome and reduce cancer-associated mortality in CVID.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. These data can be found here: BioProject PRJNA750325 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA750325).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hannover Medical School. The patients/participants provided their written informed consent to participate in this study (ethics vote number: 5582).

AUTHOR CONTRIBUTIONS

Research design: GS and FA; sample collection GS, GA, SH and VP; tNGS data analysis: GS and FA; WES analysis: GS, SvH and GSch; functional assay for CTLA-4 insufficiency, IRA; data analysis: LB, VP, ES, GS and FA; funding acquisition: GS, TW, RS, SvH; writing and contributing to writing of the manuscript GS, FA and all authors. All authors contributed to the article and approved the 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.2022.742530/full#supplementary-material

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Liver Stiffness by Transient Elastography Correlates With Degree of Portal Hypertension in Common Variable Immunodeficiency Patients With Nodular Regenerative Hyperplasia

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Nodular regenerative hyperplasia (NRH) is associated with high morbidity and mortality in patients with common variable immunodeficiency (CVID). While liver biopsy is the gold standard for NRH diagnosis, a non-invasive technique could facilitate early disease recognition, monitoring, and/or immune intervention. We performed a cross-sectional analysis of ultrasound-based transient elastography (TE) in patients with CVID to evaluate liver stiffness and compared this between patients with (N = 12) and without (N = 6) biopsy-proven NRH. Additionally, these data were compared to a cohort followed at our institution for non-alcoholic fatty liver disease (NAFLD) (N = 527), a disease for which TE has routine diagnostic use. Clinical and pathologic features of NRH were evaluated as correlates of liver stiffness, and receiver operating characteristic curves were used to define a liver stiffness cutoff with diagnostic utility for NRH among CVID patients. CVID patients with NRH had a more severe disease presentation compared to those without. This included increased autoinflammatory disease comorbidities, combined B-cell and T-cell dysfunction, and abnormal liver biochemistries (specifically an increased mean alkaline phosphatase level [proximal to TE, 250 vs. 100 U/L; p = 0.03; peak, 314 vs. 114 U/L; p = 0.02). Results of TE demonstrated a significantly elevated liver stiffness in CVID patients with NRH (mean 13.2 ± 6.2 kPa) as compared to both CVID patients without NRH (mean 4.6 ± 0.9 kPa) and non-CVID patients with NAFLD (mean 6.9 ± 5.5 kPa) (p < 0.01).

No single or composite histopathologic feature of NRH correlated with liver stiffness including nodule size, nodule density, sinusoidal dilation, fibrosis, and/or lymphocytosis. In contrast, liver stiffness by TE was significantly correlated with clinical parameters of portal hypertension, including an elevated hepatic venous pressure gradient, an increased splenic longitudinal diameter, presence of varices, and presence of peripheral edema. A liver stiffness of greater than or equal to 6.2 kPa was a clinically significant cutoff for NRH in CVID patients. We propose that TE has diagnostic utility in CVID, particularly in the presence of immunophenotypic features such as combined B-cell and T-cell dysfunction, autoinflammatory comorbidities, and/or abnormal liver tests. Elevated liver stiffness by TE should raise suspicion for NRH in patients with CVID and prompt expedited evaluation by hepatology.

Keywords: common variable immunodeficiency (CVID), nodular regenerative hyperplasia (NRH), transient elastography (TE), fibroscan©, liver disease, liver biopsy

INTRODUCTION

Common variable immunodeficiency (CVID) is the most frequent symptomatic antibody deficiency in adults (1). It comprises a heterogeneous group of disorders with increased infectious risk resulting from impaired and dysregulated immunity. Diagnosis requires low immunoglobulin (Ig) G combined with low IgA or IgM, impaired vaccine response, and the exclusion of secondary causes. While patients typically present with recurrent infections, more than 30% additionally demonstrate non-infectious manifestations (2). Liver disease is a frequent and underrecognized complication in patients with CVID and can be a consequence of recurrent infectious insults, malignancy, and/or immune dysregulation impacting the liver. The most prevalent liver pathology in patients with CVID is nodular regenerative hyperplasia (NRH), which affects 41%–87% of patients with CVID who undergo liver biopsy (3–6). NRH is believed to be mediated through T-cell infiltration of the sinusoidal endothelium, causing intra-hepatic vasculopathy. This process leads to hepatocyte damage, regeneration, and the characteristic nodular appearance of the liver parenchyma (4). Clinically, it manifests with a frequently asymptomatic rise in alkaline phosphatase (ALP), with or without changes in aspartate transaminase (AST) or alanine transaminase (ALT). These changes may be present for years, followed by jaundice, portal hypertension, and varices *via* nodular compression of sinusoids, portal, and central vasculature (7, 8). Given the association of NRH with portal hypertension, it follows that CVID patients with NRH also demonstrate increased morbidity and mortality as compared to the general CVID population (2, 9, 10). Currently, there are no Food and Drug Administration (FDA)-approved treatment modalities for NRH in CVID patients, although biologics are being tried for the treatment of various autoinflammatory end-organ complications in primary immunodeficiencies, making early diagnosis and potential early intervention the goal in CVID patient management (10–13).

The gold standard for diagnosis of NRH is a liver biopsy. In patients with CVID, NRH is characterized histologically by a nodular pattern of alternating areas of hepatic plate expansion and atrophy. NRH-like changes are often accompanied by other histologic features of CVID such as a sinusoidal lymphocytic infiltrate, mild portal and lobular inflammation, and variably prominent sinusoidal fibrosis (6). NRH-like changes may be present on liver biopsy even when clinical manifestations are subtle (such as mildly elevated liver enzymes), making improved diagnostic modalities essential. Furthermore, delays in liver biopsy procedures are common in patients with CVID due to 1) the aforementioned subtle presentation of NRH and 2) avoidance of high-risk procedures in immunodeficient patients (7). Therefore, patients frequently present with advanced disease with 19%–50% demonstrating manifestations of portal hypertension, such as gastroesophageal varices or cirrhosis, at the time of diagnosis (5, 8, 10).

Imaging modalities investigated to date for the diagnosis of NRH among CVID patients include CT, MRI, and ultrasound (US) (14, 15). Specific advantages of US imaging include being fast, low-cost, non-invasive, and in line with the goal to reduce repeat exposure to radiation in CVID patients, who are already at higher risk for both hematologic and solid-organ malignancies as compared to the general population (16). Traditionally, US with Doppler has been employed in patients with liver disease; however, non-specific findings limit its utility, and the detection of portal hypertension and splenomegaly is a late-stage finding. Vibration-controlled transient elastography (TE) or FibroScan® uses transducer-induced vibrations to create shear waves that move throughout the liver parenchyma. Calculations of the speed of these shear waves can estimate the degree of liver stiffness (17). This approach has been well validated in chronic viral hepatitis to detect cirrhosis and stratify risk for portal hypertension and varices (18, 19). Additional data have demonstrated significant clinical utility in the diagnosis of non-alcoholic fatty liver disease (NAFLD) (20, 21). TE has

been studied, generally, in NRH with variable results (14). More recently, this modality has demonstrated promise in the non-invasive detection of liver disease in patients with CVID, particularly those with lymphoproliferative and enteropathy phenotypes (22).

Given the prevalence of NRH in CVID patients with liver disease, we hypothesized that TE may be a non-invasive imaging tool that has diagnostic utility. In this study, we performed TE among CVID patients and analyzed liver stiffness by kPa, comparing groups with and without biopsy-proven NRH. In addition, we analyzed pathologic and clinical features, such as the severity of portal hypertension, which correlated with the degree of liver stiffness by TE among CVID patients. Finally, we created and analyzed receiver operating characteristic (ROC) curves to define a liver stiffness cutoff by TE with diagnostic utility for NRH among CVID patients.

METHODS

Participants

This study was performed at Mass General Brigham under an Institutional Review Board-approved protocol (#2011P000940).

Participants were recruited from Massachusetts General Hospital Immunology and Gastroenterology clinics from January 1, 2018, to March 14, 2022. CVID was diagnosed using International Consensus Document (ICON) criteria (1). NAFLD was diagnosed using the American Association for the Study of Liver Diseases (AASLD) guidelines (23). In a CVID cohort followed up longitudinally at our single-center institution, a consecutive sample of CVID patients with abnormal liver biochemistries (AST > 40 U/L, ALT > 55 U/L, or ALP > 100 U/L) and biopsy-proven NRH were offered TE (N = 12). CVID patients without NRH (defined as no abnormal liver biochemistries (N = 4) or abnormal liver biochemistries biopsied negative for NRH (N = 2)) were offered TE. At the time of initial enrollment, CVID participants had not received active immunosuppressive therapy for an end-organ lymphoinfiltrative disease related to CVID. One CVID patient with NRH did receive a single dose of abatacept therapy (500 mg intravenously) 1 month prior to the time of TE. One CVID patient with NRH was on chronic mycophenolate mofetil (1,000 mg twice daily) for neuromyelitis optica. The remainder of the CVID participants received no B-cell or T-cell suppressive therapy in the 6 months prior to the time of TE. A total of N = 527 patients with NAFLD were included in a comparator cohort. Exclusion criteria were any evidence of hepatitis virus infection (defined as a positive viral load by PCR), alcoholic cirrhosis, and/or moderate ascites at the time of imaging. Additionally, in the NAFLD cohort, previously diagnosed autoimmune hepatitis was an exclusion criterion.

Measurement of Liver Stiffness

TE using FibroScan® was performed by a trained ultrasonographer who completed 10 serial measurements with a median score reported. The raw median score in kPa was used for all subsequent analyses. In CVID patients, a ROC curve was generated to define the most accurate diagnostic kPa cutoff for NRH. A cutoff of ≥7.5 kPa was also assessed given its clinical significance in determining elevated liver stiffness among patients diagnosed with NAFLD (24). Controlled attenuation parameter (CAP) values (dB/m) were evaluated to quantify the relationship between liver stiffness and hepatic steatosis in those patients (N = 10) who had this value calculated.

Liver Histopathology

Liver biopsy slides directly available at our institution were included in the histopathologic analysis (N = 7) and were reviewed by a gastrointestinal (GI) pathologist blinded to the TE data and the initial pathology report. H&E and trichrome-stained slides were reviewed for each case. Histopathology was deemed to be generally consistent, or not, with NRH and additionally was classified based on the following characteristics: the number of nodules identified (N), diameter of the largest nodule (mm), nodule length (mm), nodule density (nodules/10 mm of core length), thickest hepatic plate (N cell layers), sinusoidal dilatation (±), sinusoidal lymphocytosis (±), centrilobular fibrosis, and/or portal fibrosis. Fibrosis was graded as absent, focal, diffuse, bridging, or cirrhosis.

Collection and Definition of Clinical and Laboratory Data

Participants' clinical data were extracted from the electronic medical record. For all CVID participants, this included a review of complete blood count (CBC) with differential and liver biochemistries (AST, ALT, ALP, gamma-glutamyl transferase (γ-GGT), total bilirubin, albumin, coagulation factors (prothrombin/partial thromboplastin time), and ammonia). Peak ALP was also recorded, included in the absence of other acute causes of elevation (e.g., infection and acute clinical decompensation). Patient immunophenotype was reviewed including immunoglobulin levels, peripheral flow cytometry including T-cell, B-cell, NK-cell, class-switched memory B-cell, and naïve/memory T-cell counts (absolute and relative percentages in peripheral blood) closest to the time of TE measurement. T-cell proliferation to mitogens, antigens, and anti-CD3 were also reviewed. Clinical parameters of liver disease were reviewed by the electronic medical recorded diagnosis, as follows: the presence of clinical portal hypertension, varices (grade 1–3), ascites (trace only), and/or peripheral edema. In individuals with transjugular liver biopsies, hepatic venous pressure gradient (HVPG) measurements were evaluated (N = 8). HVPG >5 and >10 mmHg were used to define any portal hypertension and clinically significant portal hypertension, respectively (25). In CVID participants with abdominal CT imaging available (N = 13), spleen size was measured using the largest anterior–posterior diameter on axial imaging, with those ≥12 cm considered enlarged (26). Four CVID participants had prior splenectomy and were excluded from this portion of the analysis. Prior infections specifically reviewed included hepatitis viruses (hepatitis A, B, and C), Epstein–Barr virus (EBV), cytomegalovirus (CMV), and

Giardia. Autoinflammatory CVID complications were defined as the presence of autoimmune enteropathy, autoimmune cytopenias, chronic (>6 months) lymphadenopathy, and/or granulomatous–lymphocytic interstitial lung disease (GLILD). Route of replacement immunoglobulin and dose by body weight were also recorded.

CVID was subcategorized as complicated or uncomplicated. Complicated CVID was defined by the presence of any autoinflammatory clinical feature (described above) or the presence of a combined deficiency immunophenotype (class-switched memory B cells <2% of total CD19+ B cells and naïve CD4+CD45RA+ T cells <20% of total CD4+ T cells).

For all data recorded above, the measurement closest to the time of the TE was recorded. The median time from TE to liver biopsy was 359 days (Q1–Q3, 163–890 days), to immunoglobulin level was 162 days (72–408 days), to flow cytometry was 185 days (78–675 days), to liver biochemistry was 39 days (15–147 days), and to CBC was 49 days (28–112 days).

Statistical Analysis

Data are represented as means ± SD, median (Q1–Q3), or proportions unless otherwise noted. The relationship between liver stiffness with immunologic and clinical variables was measured using one-way ANOVA with Tukey's post-hoc correction and simple linear regression. A Mood's median test compared median liver stiffness measurements. The chi-square test and logistic regression were used to compare categorical data. To account for the small sample size, log transformation was applied to continuous variables and Fisher's exact test to categorical variables when estimating p-values. If linear regression assumptions were not met after transformation, Spearman's correlation was performed. Figures were created utilizing GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) or BioRender.com. Statistical analyses were completed with SAS 9.4 (SAS Institute, Cary, NC, USA); a two-tailed p-value of <0.05 was considered significant.

RESULTS

Characteristics of Patients Who Underwent Transient Elastography

TE was performed at our single-center institute on 12 CVID patients with NRH, 6 CVID patients without NRH, and 527 non-CVID patients with NAFLD. The median age of all participants was 55 (Q1–Q3, 46–64) years, and 55.7% were female. There were no significant differences in age, race, and sex in the subgroups of CVID with NRH, CVID without NRH, and non-CVID with NAFLD. Within CVID subgroups, there was no significant difference between age at diagnosis and time from diagnosis to completion of liver stiffness measurement (**Supplementary Table 1**).

Immunophenotypes of CVID patients with and without NRH who underwent TE were evaluated (**Supplementary Table 2**). CVID patients with NRH had lower naïve CD4+CD45RA+ T cells, by both absolute count and percentage (84 vs. 276, cells/μl, 18% vs. 43%; p = 0.04). There were no significant differences in the remainder of lymphocyte subsets, immunoglobulin levels, or T-cell functional studies analyzed, although there was a trend towards lower class-switched memory B cells in CVID patients with NRH (1.4% vs. 3.1%; p = 0.11).

Clinical complications and immunoglobulin treatment differences among CVID patients with and without NRH who underwent TE were compared (**Supplementary Table 3**). A larger proportion of CVID patients with NRH had autoinflammatory complications compared to CVID patients without NRH (83% vs. 17%; p = 0.01). Specifically, CVID patients with NRH more often had additional diagnoses of GLILD and lymphadenopathy as compared to CVID patients without NRH (67% vs. 0%; p = 0.01, 58% vs. 0%; p = 0.04, respectively). In addition, there was a trend toward a higher dose per body weight of replacement immunoglobulin in those with NRH, compared to those without (mean 699 vs. 485, mg/kg/month; p = 0.05). Consistent with these immunophenotypic and clinical data, all (N = 12) CVID patients with NRH met the classification for complicated CVID, while only 1 of 6 CVID patients without NRH similarly met the classification for complicated CVID (**Supplementary Table 1**).

Finally, we compared markers of liver disease in CVID patients with and without NRH who underwent TE (**Supplementary Table 4**). We identified higher levels of AST (54 vs. 26, U/L; p < 0.01), ALP proximal to TE (250 vs. 100, U/L; p = 0.03), peak ALP (314 vs. 114, U/L; p = 0.02), albumin (4.3 vs. 3.9, g/dl; p = 0.02), and total bilirubin (0.75 vs. 0.35, mg/dl; p = 0.01) in CVID patients with diagnosed NRH compared to those without NRH. Several markers of portal hypertension additionally were associated with diagnosed NRH, including increased splenic longitudinal diameter (16.6 vs. 11.2, cm; p = 0.02), presence of any grade varices (67% vs. 0%; p = 0.04), and clinical portal hypertension diagnosed in the electronic medical record (83% vs. 0%; p < 0.01).

Patients With Common Variable Immunodeficiency and Nodular Regenerative Hyperplasia Have Elevated Liver Stiffness by Transient Elastography

Next, we compared measures of liver stiffness by TE among CVID patients with and without NRH. As an additional disease comparator, we included a cohort of patients followed up at our center for NAFLD, a disease where TE is already used in routine clinical diagnosis. We identified a significantly higher measure of liver stiffness in CVID patients with NRH compared to CVID patients without NRH and non-CVID patients with NAFLD (**Figure 1**). CVID participants with diagnosed NRH had a mean liver stiffness of 13.2 (± 6.2) kPa and a median liver stiffness of 11.9 (8.4–18.1) kPa. In contrast, CVID participants without NRH had a mean liver stiffness of 4.6 (± 0.9) kPa and median liver stiffness of 4.6 (4.1–5.3) kPa (p = 0.01, CVID with NRH vs. without NRH). Finally, non-CVID NAFLD patients had a mean liver stiffness of 6.9 (± 5.5) kPa and median liver stiffness of 5.5 (4.3–7.1) kPa (p < 0.01, CVID with NRH vs. NAFLD). Liver stiffness was positively correlated with CAP measurements,

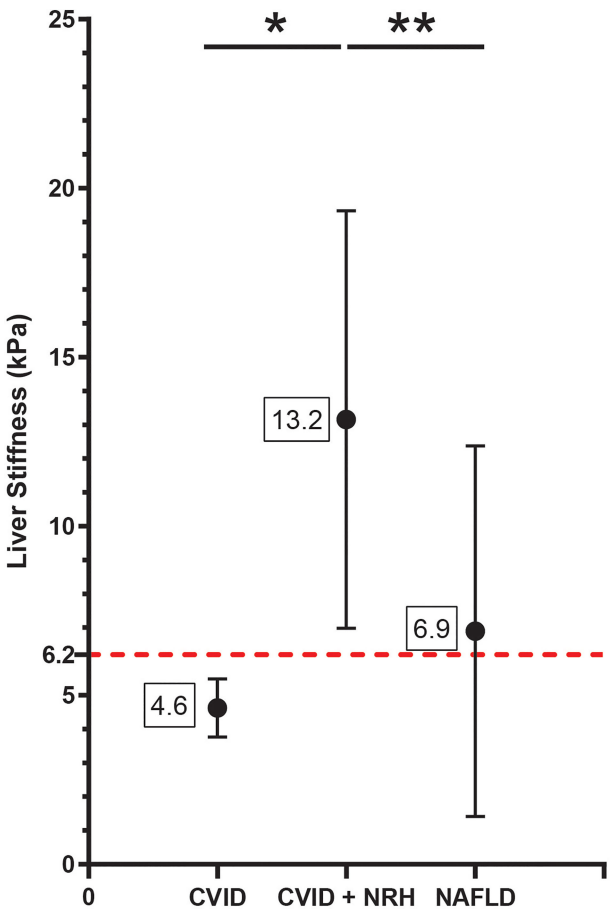


FIGURE 1 | Liver stiffness by transient elastography is significantly elevated in CVID patients with NRH. Liver stiffness measurements (kPa) by transient elastography shown as mean (± SD) in CVID patients with biopsy-proven NRH (CVID + NRH, N = 12), CVID patients without NRH (CVID, N = 6), and non-CVID patients with non-alcoholic fatty liver disease (NAFLD, N = 527). Significance by one-way ANOVA with Tukey's post-hoc correction; *, p = 0.01; **, p < 0.01. Red dotted line indicates a diagnostic cutoff value for liver stiffness of 6.2 kPa (defined using the ROC curve in **Figure 4**). CVID, common variable immunodeficiency; NRH, nodular regenerative hyperplasia; ROC, receiver operating characteristic.

although this relationship was not significant (r = 0.53; p = 0.12) (**Supplementary Figure 1**).

Liver Stiffness by Transient Elastography Does Not Correlate With Any Specific Histopathologic Feature of Nodular Regenerative Hyperplasia Among Common Variable Immunodeficiency Patients

NRH has been associated with a diverse spectrum of histopathologic findings (27, 28). A detailed review of liver histopathology was performed to determine which specific features may be driving the observed increased measure of liver stiffness in CVID patients with NRH. Using blinded scoring, we did not observe any appreciable association between liver stiffness measurements and nodule size, nodule density, fibrosis, or sinusoidal lymphocytosis (**Figure 2**). A composite score of these histopathologic variables was created to minimize redundancy and maximize concordance during interpretation. Again, there was no significant association between composite scoring and liver stiffness measurements by TE.

Liver Stiffness by Transient Elastography Significantly Correlates With Portal Hypertension Among Common Variable Immunodeficiency Patients

As we observed no association between histopathologic features and liver stiffness by TE in CVID patients with NRH, we alternatively evaluated whether the observed increased liver stiffness in this population was driven by the severity of physiologic portal hypertension.

Among all CVID participants, patients with clinical evidence of portal hypertension were found to have significantly higher liver stiffness measurements by TE. Specifically, there was a significant association between liver stiffness and splenic longitudinal diameter (r = 0.61; p = 0.03), presence of grade 1–3 varices (mean kPa 16.5 vs. 6.4, median kPa 17.6 vs. 5.8; p < 0.01),

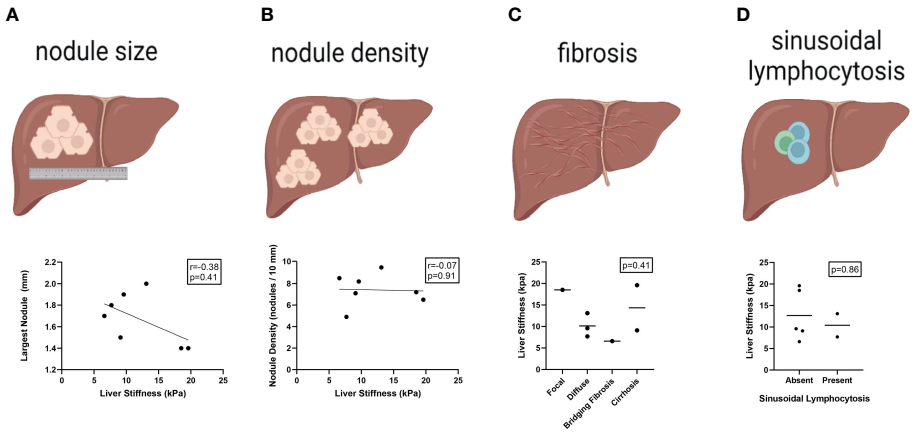


FIGURE 2 | Liver stiffness by transient elastography does not correlate with specific histopathologic features of NRH in CVID patients. Liver stiffness measurements (kPa) by transient elastography compared across histopathologic features of NRH in CVID patients with available liver biopsy (N = 7), including size of largest nodule **(A)**, nodule density **(B)**, centrilobular fibrosis **(C)**, and sinusoidal lymphocytosis **(D)**. Significance by one-way ANOVA with Tukey's post-hoc correction or Spearman's correlation (r) with significance (p) shown. Line of best fit **(A, B)** and mean value **(C, D)** are shown. NRH, nodular regenerative hyperplasia; CVID, common variable immunodeficiency.

and presence of peripheral edema (mean kPa 18.4 vs. 8.7, median kPa 19.6 vs. 6.6; p = 0.03, p=0.07, respectively). Additionally, liver stiffness was higher among CVID patients with diagnosed portal hypertension, either clinically (mean kPa 14.2 vs. 5.4, median kPa 13.3 vs. 5.1; p < 0.01) or by increased HVPG (mean kPa 15 vs. 7.7, median kPa 13.1 vs. 5.5; p < 0.01) (**Figure 3**, **Table 1**). In contrast, CAP measurements by TE did not significantly differ across clinical parameters of portal hypertension (**Supplementary Table 5**).

Given the higher prevalence of complicated CVID in patients with versus without NRH who underwent TE, we evaluated if other clinical parameters correlated with the measure of liver stiffness in this patient demographic. The presence of CVID-related autoinflammatory disease was not associated with higher liver stiffness by TE. Specifically, there was no difference in a mean or median liver stiffness between CVID patients with GLILD, lymphadenopathy, enteropathy, or cytopenias. Additionally, liver stiffness values did not differ based on the history of GI infection or type of replacement immunoglobulin received (**Table 1**).

Liver Stiffness and Alkaline Phosphatase Have Utility in the Diagnosis of Nodular Regenerative Hyperplasia Among Common Variable Immunodeficiency Patients

Previously, an ALP level in peripheral blood >1.5 times the upper limit of normal was suggested as a useful marker in the diagnostic workup of NRH for CVID patients, specifically to prompt further liver biopsy (7). We sought to comparatively analyze the diagnostic utility of liver stiffness by TE versus ALP in our cohort of CVID patients with and without NRH. We created ROC curves for liver stiffness (kPa) and ALP (proximal to the time of TE and peak level) (**Figure 4**). Liver stiffness had robust ROC curve parameters, with an area under the curve (AUC) of

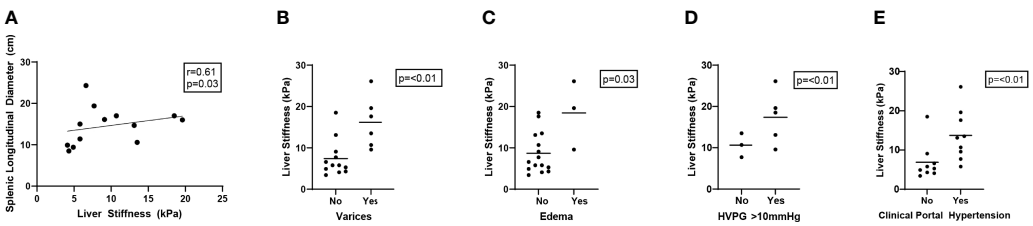


FIGURE 3 | Liver stiffness by transient elastography correlates with clinical parameters of portal hypertension in CVID patients. Liver stiffness measurements (kPa) by transient elastography compared across clinical parameters of portal hypertension in CVID patients, including splenic longitudinal diameter (**A**, N = 13 scored), presence of varices (grade 1–3) (**B**, N = 18 scored), presence of peripheral edema (**C**, N = 18 scored), elevated (>10 mmHg) hepatic venous pressure gradient (HVPG) (**D**, N = 8 scored), and clinically diagnosed portal hypertension in the electronic medical record based on any combination of these data (**E**, N = 18 scored). Significance by one-way ANOVA with Tukey's post-hoc correction or Spearman's correlation (r) with significance (p) shown. Line of best fit **(A)** and mean value **(B–E)** are shown. CVID, common variable immunodeficiency.

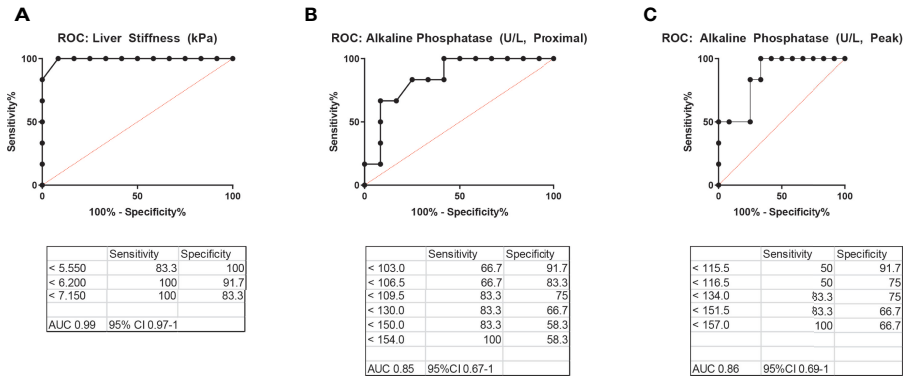
TABLE 1 | Liver stiffness by transient elastography correlates with clinical markers of portal hypertension.

	kPa (mean)	p-Value	kPa (median)	p-Value
Clinical parameters of portal hypertension				
Splenic longitudinal diameter				
≥12 cm	12.1		13.1	
<12 cm	5.8	0.04	5.3	0.11
Splenectomy				
Yes	14.5		12.1	
No	9.1	0.13	6.2	0.27
Hepatic venous pressure gradient				
>5 mmHg	15		13.1	
≤5 mmHg	7.7	<0.01	5.5	<0.01
>10 mmHg	17.4		18.5	
≤10 mmHg	7.9	<0.01	5.5	0.02
Varices				
Yes	16.5		17.6	
No	6.4	<0.01	5.8	<0.01
Ascites				
Yes	16.4		13.5	
No	9.1	0.08	6.6	0.07
Edema				
Yes	18.4		19.6	
No	8.7	0.03	6.6	0.07
Clinical portal hypertension				
Yes	14.2		13.3	
No	5.4	<0.01	5.1	<0.01
Autoinflammatory comorbidity				
Yes	11.3		9.6	
No	8.8	0.22	5.3	0.16
GLILD				
Yes	11.1		9.4	
No	9.7	0.41	5.5	0.36
Lymphadenopathy				
Yes	12.4		10.7	
No	9.0	0.17	5.8	0.16
Enteropathy				
Yes	13.1		10.2	
No	9.5	0.36	7.2	0.27
Cytopenia				
Yes	10.3		10.4	
No	10.3	0.78	7.9	1
Gastrointestinal infection				
Yes	12.6		9.2	
No	9.8	0.47	7.9	1
Replacement immunoglobulin				
SCIG	9.2		5.8	
IVIG	11	0.54	9.1	0.64

CVID, common variable immunodeficiency; NRH, nodular regenerative hyperplasia; kPa, kilopascal; GLILD, granulomatous lymphocytic interstitial lung disease; SCIG, subcutaneous immunoglobulin; IVIG, intravenous immunoglobulin.

0.99 (95%CI 0.97–1) and a cutoff of 6.2 kPa demonstrating excellent performance by sensitivity and specificity for prediction of NRH in CVID. ROC curves for proximal ALP and peak ALP were both adequate diagnostic tests, albeit with lower AUCs of 0.85 (95%CI 0.67–1) and 0.86 (95%CI 0.69–1), respectively. An ALP of 154 U/L (proximal) and 157 U/L (peak) had a sensitivity of 100% for the detection of NRH in CVID, although lacked specificity (58% and 67%, respectively).

Finally, we analyzed peripheral blood immunophenotypes and liver biochemistries, comparing the liver stiffness cutoffs of 6.2 kPa (defined by our ROC above) and 7.5 kPa [a threshold for increased liver stiffness previously used in NAFLD (24)] (**Tables 2**, **3**). CVID patients with ≥6.2 kPa had significantly lower mean absolute CD4+CD45RA+ naïve T cells (82 vs. 241 cells/μl; p = 0.04), higher AST levels (53 vs. 33 U/L; p = 0.04), and higher ALP levels (proximal, 259 vs. 107 U/L; p = 0.04; peak, 328 vs. 121 U/L; p = 0.01). The only significant comparisons using a cutoff of ≥7.5 kPa were albumin, which was lower (3.8 vs. 4.3 mg/dl) in the high kPa group, and peak ALP, which was higher (335 vs. 138 U/L) in the high kPa group. Otherwise, there were no significant differences in immunoglobulin levels, lymphocyte subsets, or liver biochemistries between the two groups.



	Sensitivity	Specificity
< 5.550	83.3	100
< 6.200	100	91.7
< 7.150	100	83.3
AUC 0.99	95% CI 0.97-1	

	Sensitivity	Specificity
< 103.0	66.7	91.7
< 106.5	66.7	83.3
< 109.5	83.3	75
< 130.0	83.3	66.7
< 150.0	83.3	58.3
< 154.0	100	58.3
AUC 0.85	95%CI 0.67-1	

	Sensitivity	Specificity
< 115.5	50	91.7
< 116.5	50	75
< 134.0	83.3	75
< 151.5	83.3	66.7
< 157.0	100	66.7
AUC 0.86	95%CI 0.69-1	

FIGURE 4 | Receiver operating characteristic (ROC) curves for the diagnosis of NRH in CVID patients. ROC curves for **(A)** liver stiffness by transient elastography, **(B)** alkaline phosphatase (ALP) level in peripheral blood most proximal to the time of transient elastography, and **(C)** peak ALP level in peripheral blood, excluding acute illness, to diagnose NRH in patients with CVID. AUC, area under the curve; NRH, nodular regenerative hyperplasia; CVID, common variable immunodeficiency.

Together, these data suggest that a liver stiffness measurement by TE of ≥6.2 kPa is an accurate diagnostic cutoff for NRH in patients with CVID. We developed a clinical algorithm for the early detection of NRH in patients with CVID utilizing this diagnostic cutoff (**Figure 5**).

DISCUSSION

This study demonstrates the utility of TE in diagnosing NRH among patients with CVID. While prior investigations have analyzed the relationship between TE and liver disease in CVID, this is the first study to our knowledge to do so in those with biopsy-confirmed NRH.

It has been established that liver stiffness measurements are elevated in CVID-related liver disease. Crescenzi et al. previously investigated TE in CVID patients with liver disease demonstrating a mean liver stiffness of 7.5 kPa, with 75% of participants meeting the definition of complicated CVID used in this study. In the absence of enteropathy or polyclonal lymphoproliferation, the mean liver stiffness appeared to be below 6.2 kPa (22). Prior studies have also demonstrated an association of elevated liver stiffness with CVID phenotypes such as polyclonal lymphoproliferation (GLILD, persistent lymphadenopathy, and granuloma) and enteropathy, as well as markers of portal hypertension (e.g., splenic longitudinal diameter) (5, 7, 22). The most established predictor of NRH in CVID is an elevated ALP level, although any of the liver biochemistry tests may be abnormal. Ward et al. eloquently demonstrated that ALP levels in NRH can follow several different patterns, the most common being a steady increase over time, and this generally starts several years after CVID diagnosis (7). Importantly, 30% or more of patients with liver disease in CVID may have normal liver biochemistries (29). It is known that liver

TABLE 2 | Relationship between peripheral blood immunophenotypes and liver stiffness cutoffs for NRH in CVID patients.

	kPa ≥ 6.2	kPa < 6.2	p-Value	kPa ≥ 7.5	kPa < 7.5	p-Value
Immunoglobulins [mean (mg/dl)]						
IgG	1038	943	0.63	1029	967	0.50
IgA	39	85	0.19	42	76	0.29
IgM	315	43	0.17	323	66	0.35
Flow cytometry [mean (cells/μl), %]						
CD3+	1319, 72	1041, 70	0.71	1401, 73	973, 69	0.99
CD4+	857, 46	770, 51	0.47	917, 47	708, 47	0.80
CD8+	407, 21	239, 17	0.87	428, 21	232, 17	0.77
CD3−CD16+56+	188, 11	181, 12	0.42	175, 8	199, 15	0.21
CD4+CD45RA+	82, 17	241, 38	0.043	91, 19	201, 32	0.54
CD4+CD45RO+	530, 77	318, 55	0.88	560, 75	308, 62	0.78
CD8+CD45RA+	136, 54	167, 66	0.234	148, 59	148, 57	0.96
CD8+CD45RO+	92, 36	62, 27	0.84	87, 32	74, 34	0.77
CD19+	616, 15	223, 16	0.92	676, 16	197, 14	0.73
CD19+CD27+	17, 9	54, 21	0.14	18, 7	47, 23	0.18
CD19+CD27+IgM/IgD−	1.7, 1.5	6.5, 2.6	0.28	1.8, 0.8	5.7, 3.4	0.33

Significance by one-way ANOVA shown.
CVID, common variable immunodeficiency; NRH, nodular regenerative hyperplasia; kPa, kilopascal.
**T-cell function excluded as N = 6–12 missing for each variable.*

TABLE 3 | Relationship between liver biochemistries and liver stiffness cutoffs for NRH in CVID patients.

	kPa ≥ 6.2	kPa < 6.2	p-Value	kPa ≥ 7.5	kPa < 7.5	p-Value
Liver biochemistries*						
AST (U/L)	53	33	0.04	52	36	0.12
ALT (U/L)	43	30	0.32	44	30	0.31
ALP (U/L, proximal)	259	107	0.04	259	126	0.1
ALP (U/L, peak)	328	121	0.01	335	138	0.03
γ-GGT (U/L)**	151	518	0.18	293	168	0.66
Albumin (g/dl)	3.9	4.2	0.1	3.8	4.3	0.01
Total bilirubin (mg/dl)	0.73	0.44	0.08	0.76	0.43	0.05
PT/PTT						
Abnormal (%)	9	0	1	10	0	1

Significance by one-way ANOVA shown.
CVID, common variable immunodeficiency; NRH, nodular regenerative hyperplasia; kPa, kilopascal.
**Ammonia level not collected on any participants.*
***γ-GGT, N = 7 participants with data.*

disease in CVID is a poor prognostic indicator and that NRH specifically is associated with portal hypertension complications such as hemoptysis and ascites (4, 5, 22).

In this study, we identified significantly elevated liver stiffness by TE in CVID patients with NRH compared to both CVID patients without NRH and non-CVID patients with NAFLD. Interestingly, we did not find a statistically significant association between histopathologic features of NRH on liver biopsy and liver stiffness by TE in our CVID patient demographic. This is consistent with other studies of NRH in non-CVID populations, where liver stiffness by TE has not been associated with specific histopathologic features (14). This lack of concordance between NRH histopathology on biopsy and liver stiffness by TE could be due to multiple factors including limited consensus criteria in the histopathologic definition of NRH or adequacy of biopsy samples (28). In contrast, we did demonstrate a significant association between liver stiffness by TE and several clinical measures of portal hypertension in this study. These data suggest that TE has specific utility in identifying progression to portal hypertension among CVID patients with NRH, which is of great clinical importance given the concomitant increase in CVID patient morbidity and mortality associated with this clinical progression (4, 22).

There are several limitations and sources of bias to consider in this study. While all eligible CVID participants with NRH were recruited, not all of those without NRH participated in the study.

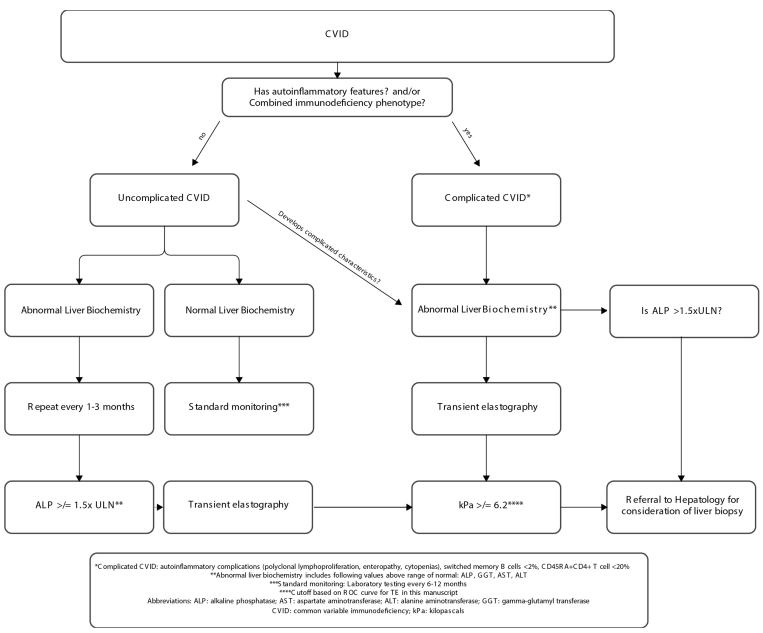


FIGURE 5 | Clinical algorithm for early detection of nodular regenerative hyperplasia in individuals with CVID. CVID, common variable immunodeficiency; ULN, upper limit normal

This may introduce selection bias in that CVID patients without NRH who consented to be included may be different from those who did not. This study is also cross-sectional in nature, and liver stiffness levels were not followed up over time, limiting causal inference. Furthermore, the study population was relatively small with recruitment from an academic tertiary care center, limiting statistical power and adding to potential selection bias or increasing type I error. Given the rarity of CVID, though, this is not unexpected and is a difficult limitation to overcome. It is important to note that four participants had splenectomy before this study, and thus, splenic diameter measurements were unavailable. As those with prior splenectomy likely had severe disease, censoring these patients in our analysis would have biased results towards the null, minimizing an association. While we did incorporate a comparison between liver stiffness by TE in those with CVID and NAFLD, mean liver stiffness measurements in NAFLD participants were below the established threshold of clinically significant fibrosis. We were unable to correlate liver stiffness measurements in those with NAFLD to the extent of fibrosis on biopsy given our data set. The influence of steatosis and other pathologic processes unrelated to NRH on liver stiffness measurements is difficult to measure and must be acknowledged as well. We attempted to correlate CAP as a surrogate of hepatic steatosis to liver stiffness and markers of portal hypertension, and while there did seem to be a positive correlation, this was not statistically significant, which we believe was due to the small sample size. Finally, most participants with NRH in this study had severe disease, with evident portal hypertension. It is therefore difficult to make conclusions related to the utility of TE as a predictor of early NRH. Future studies investigating the impact of early NRH diagnosis and the impact of underlying complicated CVID features in those without liver disease will be of great importance.

The current standard of care in patients with CVID and NRH suffers from diagnostic delays and treatments that carry high morbidity and mortality (i.e., transplant) (10, 11). TE is a potentially helpful tool for the diagnosis and monitoring of liver disease through the progression of disease and response to treatment in NRH. Based on the findings in this study, we propose an algorithm that utilizes CVID immunophenotype, liver biochemistries, and liver stiffness by TE to stratify NRH risk among CVID patients. Specifically, for patients with features consistent with complicated CVID, we proposed that any abnormal liver biochemistry measurement should trigger the measurement of liver stiffness by TE. Furthermore, the patient should be referred to hepatology for consideration of liver biopsy if either of the following criteria are met: the measure of liver stiffness by TE is at or above 6.2 kPa or the ALP level (peak) is >1.5× the upper limit of normal (**Figure 5**). Future prospective studies are needed and should incorporate individuals with elevated ALP and uncomplicated CVID. Recognition of early disease, as well as confirmatory biopsy, has the potential to improve our ability to recognize and/or prognosticate regarding the timing of complications, such as portal hypertension, in CVID patients with NRH. As immunomodulatory therapies become increasingly available for the treatment of immune dysregulation in CVID, there is an opportunity for improved treatment and management as our understanding of the disease process that leads to elevated liver stiffness in NRH improves.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was performed at Mass General Brigham under an Institutional Review Board-approved protocol (#2011P000940). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DD wrote the manuscript and performed the chart review and statistical analyses on the cohort. JS, PB, and KC provided patients from the gastroenterology clinic, as well as clinical expertise. RC reviewed liver biopsy histopathology and provided expertise in the creation of biopsy scoring for NRH. NY performed the chart review. JF conceived the project and supervised all aspects of the work. SB and JF provided expertise in immunology. All authors reviewed the manuscript, 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.2022.864550/full#supplementary-material

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