Diagnostic Yield and Therapeutic Consequences of Targeted Next-Generation Sequencing in Sporadic Primary Immunodeficiency

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Keywords

Primary immunodeficiency · Inborn errors of immunity · Common variable immunodeficiency · Unclassified antibody deficiency · Combined immunodeficiency · Primary antibody deficiencies

Abstract

Introduction: Primary immunodeficiencies (PIDs) are a heterogeneous group of disorders characterized by increased susceptibility to infections, immune dysregulation, and/or malignancy. Genetic studies, especially during the last decade, led to a better understanding of the pathogenesis of primary immunodeficiencies and contributed to their classification into distinct monogenic disorders falling under one of the >430 currently known inborn errors of immunity (IEI). The growing availability of molecular genetic testing resulted in the increasing identification of patients with IEI. Here, we evaluated the diagnostic yield and the clinical consequences of targeted next-generation sequencing (tNGS) in a cohort of 294 primary immunodeficiency patients, primarily consisting of cases with sporadic primary antibody

deficiency. **Method:** We have custom designed a tNGS panel to sequence a cohort of PID patients. Agilent's HaloPlex Target Enrichment System for Illumina was used for DNA target enrichment. **Results:** tNGS identified a definite or predicted pathogenic variant in 15.3% of patients. The highest diagnostic rate was observed among patients with combined immunodeficiency or immune dysregulation, for whom genetic diagnosis may affect therapeutic decision-making. **Conclusion:** Next-generation sequencing has changed diagnostic assignment and paved the way for targeted therapeutic intervention with agents directed at reverting the disease-causing molecular abnormality or its pathophysiological consequences. Therefore, such targeted therapies and identifying the genetic basis of PID can be essential for patients with manifested immune dysregulation as conventional immunomodulatory regimens may exert an immunosuppressive effect, aggravating their immunodeficiency or may only inadequately control autoimmune or lymphoproliferative manifestations.

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Introduction

Primary immunodeficiencies (PIDs) or inborn errors of immunity (IEI) are heterogeneous disorders caused by a deficiency or a defect in one or more components of the immune system [1, 2]. So far, pathogenic variants in >430 genes have been reported to cause PID. However, the strictly monogenic aetiology of PID seems questionable when considering the incomplete penetrance and variable expressivity of genetic variants reported as disease-causing [3]. Variable expressivity, including variable age of onset and a broad spectrum of manifestations, ranging from “infections-only” to disease complicated with immune dysregulation and/or malignancy, makes a timely diagnosis of PID difficult, which leads to an increased rate of morbidity and mortality [4]. The clinical diagnosis of PID most commonly falls under one of the primary antibody deficiencies (PADs), especially common variable immunodeficiency (CVID) [5, 6]. In the last decade, the increasing availability of next-generation sequencing (NGS) technologies led to identifying the involvement of new genes in PADs. It aided the characterization of the molecular pathways involved in human B cell development and function [2, 7, 8]. The most common symptomatic PAD, CVID [7], is still considered a largely polygenic disorder, though according to more recent reports, the proportion of monogenic forms has increased, exceeding 20% of cases [8–10].

Genetic testing is currently routinely performed in most medical centres treating PID patients, resulting in an increasing number of monogenic IEI. Consequently, several studies of cohorts of patients with the same IEI that evaluated their phenotypes and outcomes have been published over the last couple of years, for example, identifying an increased risk of malignancies or association of particular IEI with systemic rheumatic or interstitial lung disease may alter diagnostic and follow-up procedures [11–13]. Further, in case of particular monogenic defects, individualized therapies with agents tailored to revert consequences of the disease-underlying genetic defect can be considered. For example, JAK-inhibitors may help treat immune dysregulation in the context of STAT3 or STAT1 gain-of-function [14], PI3K δ -specific inhibition is effective against lymphoproliferative manifestation of the activated PI3K δ syndrome [15], and abatacept may be effective in treating enteropathy and autoimmunity in patients with CTLA4 insufficiency or LRBA deficiency [16, 17].

Overall, timely molecular diagnosis and setting-specific decisions may reduce the harms associated with

PIDs and favour a better disease outcome. Hence, we employed a targeted NGS (tNGS) approach to identify the genetic background of immunodeficiency in a cohort of 294 patients, most of whom were sporadic cases of a PAD, aiming at increasing the diagnostic yield and optimizing patients' medical care.

Materials and Methods

Study Cohort

This single-centre study included a total of 294 patients with a PID, visiting the immunology outpatient clinics of either the Department of Paediatrics ($N = 28$) or the Department of Rheumatology and Immunology ($N = 266$) of the Hannover Medical School. The patients were clinically diagnosed with PID according to the European Society for immunodeficiencies criteria [18]. All relevant clinical and immunological data were obtained from the patient's medical files. Those included serum immunoglobulins, peripheral lymphocyte immunophenotyping, patient's clinical history of infections, bronchiectasis (computed tomography-confirmed), autoimmune cytopenias, such as autoimmune haemolytic anaemia, idiopathic thrombocytopenic purpura, organ-specific autoimmunity (including vitiligo, psoriasis, insulin-dependent diabetes mellitus, autoimmune thyroiditis, primary thyroid failure, atrophic gastritis, and rheumatic disease including rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus, and seronegative arthritis diagnosed according to the American College of Rheumatology [ACR]/European League Against Rheumatism [EULAR] classification criteria), benign lymphoproliferation, granulomatous disease, enteropathy, and malignancies. Benign lymphoproliferation was defined as splenomegaly (≥ 11 cm on palpation or ultrasound, including the previous splenectomy of an enlarged spleen) and/or persistent lymphadenopathy (on palpation, ultrasound, computed tomography, or magnetic resonance scan). Granulomatous disease was defined as at least 1 biopsy-proven unexplained granuloma, excluding Crohn's disease-associated granulomas. Enteropathy included all cases of biopsy-proven 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 haematological and all other forms of cancer. All patients and their parents, in the case of paediatric patients, signed an informed consent form.

Targeted NGS

A customized panel of genes associated with PID (online suppl. Table 1; see www.karger.com/doi/10.1159/000519199 for all online suppl. material) was created with the help of Agilent's web-based SureDesign application. Blood samples were collected in the immunology outpatient clinics of Paediatrics and the Department of Rheumatology and Immunology of the Hannover Medical School. Genomic DNA was isolated from the whole peripheral blood using a QIAamp DNA Blood Midi Kit (Qiagen) and quantified by a Qubit dsDNA BR Assay Kit (ThermoFisher). DNA target enrichment was performed using the Agilent's HaloPlex Target Enrichment System for Illumina Sequencing, following the manufacturer's instructions (Agilent's user manual) and as already de-

scribed [19]. In brief, DNA was fragmented using a restriction enzyme master mix prepared following the kit's protocol, and digestion was validated by gel electrophoresis. DNA fragments were hybridized to the HaloPlex probe capture library by adding the Hybridization Master Mix and Indexing Primer Cassettes. After an incubation step, the hybridized DNA fragments were captured using HaloPlex Magnetic Beads, that is, a biotin-streptavidin system and washed. In order to create circular fragments, the ends were ligated by adding the kit's ligation master mix. Subsequently, the target libraries were amplified by polymerase chain reaction. Finally, the amplified target libraries were purified with the help of AMPure XP beads and washed in ethanol. Target enrichment was validated using an Agilent TapeStation. Samples were pooled in equimolar amounts. The libraries were subjected to denaturation by adding NaOH and diluted to a final concentration of 8–12 pM. Sequencing was performed on an Illumina MiSeq system using an Illumina v2 reagent kit, following the manufacturer's protocol. Data analysis was performed with the help of Agilent's SureCall software. Most likely disease-causing candidates were confirmed by Sanger sequencing. Familial segregation was examined when samples were available.

Variants Filtering Procedure

FastQ files were aligned to the human reference genome (UCSC hg19, GRCh37) and analysed using Agilent Technologies – Sure-Call NGS software. Variants were selected according to criteria at the variant level, including allele frequency (AF), variant annotation, and potential functional effect. Using databases of variants (e.g., dbSNP, 1000 Genomes Project, Exome Aggregation Consortium, and Genome Aggregation Database) and disease-causing variants (HGMD and OMIM), we selected all rare or private variants with an AF of <1%. Furthermore, we kept nonsense variants, variants affecting the splice site, frameshift, in-frame indels, start or stop codon changes, as well as missense variants that were predicted to be deleterious by having a combined annotation-dependent depletion (CADD) score >15 and a mutation significance cut-off score below the CADD score. Also, variants were tested by MutationTaster [20], Provean, and Human Splicing Finder [21] for the pathogenicity.

Statistical Analysis

For statistical calculation, we used GraphPad prism 5.00 (GraphPad, La Jolla, San Diego, CA, USA). Descriptive statistics are reported as the median and interquartile range in continuous variables and as counts and percentages for dichotomous variables. The χ^2 test compared categorical variables. Non-categorical variables were compared with the Mann-Whitney test. All comparisons were 2-tailed, and $p < 0.05$ was considered significant.

Results

Clinical Characterization of PID Patients

Patients' demographic data and characteristics, including the clinical diagnosis and manifestations of PID, are summarized in Table 1. Most of the 294 PID patients who underwent tNGS displayed a PAD (263/294, 89.5%), more commonly diagnosed as CVID (227/294, 77.2%).

Table 1. Characteristics of studied patients (N = 294)

	N (%)
Median age at diagnosis of PID ¹ (IQR), years	30 (18–42.3)
Age group at diagnosis of PID	
0–4 years	14 (5.1)
5–18 years	62 (22.5)
>18 years	200 (72.5)
Male sex	128 (43.5)
Consanguinity ²	7 (2.4)
Familial history of PID	34 (11.6)
Clinical diagnosis of PID	
CVID	227 (77.2)
CID	29 (9.8)
Unclassified antibody deficiency	21 (7.1)
Isolated IgG subclass deficiency	7 (2.4)
SPAD	4 (1.4)
Agammaglobulinaemia	3 (1)
Other	3 (1)
Clinical manifestations of PID	
Recurrent respiratory tract infections	278 (94.6)
Bronchiectasis	60 (20.4)
“Infections only” disease	108 (36.7)
Lymphadenopathy	44 (15)
Splenomegaly	74 (25.2)
Enteropathy	27 (9.2)
ILD	20 (6.8)
Organ-specific autoimmunity	58 (19.7)
Autoimmune cytopenia ³	44 (15)
Atopic disease ⁴	48 (16.3)
Granulomatous disease	25 (8.5)
Lymphoma	5 (1.7)
Other malignancy	24 (8.2)

CID, combined immunodeficiency; CVID, common variable immunodeficiency; ILD, interstitial lung disease; IQR, interquartile range; no, number; PID, primary immunodeficiency disorder; SPAD, specific antibody deficiency; AIHA, autoimmune haemolytic anaemia; ITP, immune thrombocytopenic purpura. ¹ Analysis based on 276/294 studied patients with the known year of diagnosis. ² Analysis included 3 patients from the same consanguineous family. ³ AIHA and/or ITP. ⁴ Atopic dermatitis and/or allergic rhinitis and/or asthma.

CVID was more commonly diagnosed in adult patients, whereas combined immunodeficiencies (CIDs) were more common among patients receiving the first diagnosis of PID in childhood (online suppl. Table 2). The vast majority of studied patients had a history of recurrent infections (278/294, 94.6%), which in most cases (170/278, 61.2%) was accompanied by at least 1 non-infectious PID-associated comorbidity. Non-infectious comorbidities were more common manifestations of immune dysregulation, including autoimmunity, lymphoprolifera-

Fig. 1. Summary of genetic findings in all sequenced PID patients ($N = 294$) as well as in the more sizeable subgroups of patients with CVID ($N = 227$), CID ($N = 29$), and unclassified antibody deficiency ($N = 21$). AD, autosomal dominant; AR, autosomal recessive; CID, combined immunodeficiency; CVID, common variable immunodeficiency; PID, primary immunodeficiency.

tive, and atopic disease. Among 294 patients, 96 (32.7%) had at least 1 autoimmune disease, and 13 (4.4%) displayed polyautoimmunity (i.e., >1 autoimmune disease). Consistent with previous studies [22, 23], idiopathic thrombocytopenic purpura was the most common autoimmune manifestation in this cohort (36/294, 12.2%), followed by autoimmune haemolytic anaemia (17/294, 5.8%) (online suppl. Fig. 1). Twenty-eight out of 294 (9.5%) patients displayed a malignant disease. Among those, 8/28 had a history of skin cancer in the form of basal cell carcinoma, 4/28 patients had B cell non-Hodgkin lymphoma, 4/28 patients had gastric adenocarcinoma, and 4/26 patients had colorectal adenocarcinoma. PID-associated clinical manifestation was similar in patients with a paediatric first diagnosis of PID (<18 years) and those diagnosed in the adulthood (online suppl. Table 2).

Identified Variants

Considering the AF and CADD and mutation significance cut-off scores of each identified variant, as described above, we identified 45/294 (15.3%) patients to harbour at least 1 predicted disease-causing variant in a PID-associated gene (Fig. 1). The majority of those (23/45) harboured a genetic variant, which has been previously reported as pathogenic (Table 2). In particular, we found 37 monoallelic variants in 35/294 (11.9%) patients. Among those, more common ones were variants in *NFKB1* (7/37 monoallelic variants in 9/294 patients), fol-

lowed by variants in *STAT3* (5/37 monoallelic variants in 6/294 patients). Other PID genes with monoallelic variants included *CTLA4*, *GATA2*, *NFKB2*, *STAT1*, *IRF2BP2*, *NFKBIA*, *TCF3*, *PIK3R1*, *SOC31*, *RELA*, *MYH9*, and *VAV1* (Fig. 2). Six biallelic variants were detected in 8/294 (2.7%) patients. Those included variants in *IGLL1*, *LRBA*, *CARMIL2*, *DCLRE1C*, and *PIK3CD* (Table 2) and were identified mainly in patients stemming from consanguineous families (5/8, 62.5%). One patient with CID had compound heterozygous mutations in *RAG1*, and a patient with X-linked agammaglobulinaemia harboured a hemizygous mutation in *BTK*. Six novel variants of uncertain significance recognized in 5 PID-candidate genes, that is, *VAV2*, *SEC61A2*, *PIK3R4*, and *USP8*, were identified in 4/294 patients. An additional patient had 2 new variants in *RELB* with a yet unknown allelic phase. Finally, 21/294 (7.1%) patients had at least 1 monoallelic variant in *TNFRSF13B* (online suppl. Table 2), the gene encoding the transmembrane activator and calcium-modulating cyclophilin ligand interactor, which is a disease-predisposing gene [57, 58]. Phenotypes of all patients identified to harbour pathogenic or likely pathogenic variants are summarized in online suppl. Table 3.

Factors Affecting the Diagnostic Yield of tNGS

Overall, genetic testing utilizing tNGS identified a likely genetic diagnosis in 15.3% of studied patients. The diagnostic yield of tNGS could be different in distinct patient subgroups. We therefore evaluated the effect of fac-

Table 2. Definite and predicted pathogenic genetic variants identified through tNGS in a cohort of (N = 294) PID patients

Pati.ID	Gene	Diagnosis	Transcript ID	Transcript variant	Protein variant	dbSNP-ID	gnomAD frequency	CADD score	PolyPhen2_Pred	SIFT_Pred	Variant ^a Ref.	Gene ^b Ref.
<i>Biallelic variants</i>												
P.204	IGLL1	Agamma-globulinaemia	NM_020070.4	c.[258_258del]; [258_258del]	p.([Gln88Asnfs*7]; [Gln88Asnfs*7])	RS532338576	0.0009198	22	NA	NA	24	
P.71	LRBA	CVID	LRG_1324	c.[767_767+9del]; [767_767+9del]	LRG_1324p1:p.?			NA	NA	NA		25
P.219	CARMIL2	CID	NC_000016.10	c.[467-1G>A]; [467-1G>A]	p.?			26	NA	NA	26	27
P.285	CARMIL2	CID	NC_000016.10	c.[467-1G>A]; [467-1G>A]	p.?			26	NA	NA	26	
P.289	CARMIL2	CID	NC_000016.10	c.[467-1G>A]; [467-1G>A]	p.?			26	NA	NA	26	
P.208	PIK3CD	CVID	NM_005026.5	c.[1654_1654delG]; [1654_1654delG]	p.([Val552Serfs*26]); ([Val552Serfs*26])			24	NA	NA	28	29
P.273	DCLRE1C (ARTEMIS)	Atypical SCID (CID)	LRG_54t1	c.[464+1G>A]; [464+1G>A]	p.?							30
P.280	DCLRE1C (ARTEMIS)	Atypical SCID (CID)	LRG_54t1	c.[464+1G>A]; [464+1G>A]	p.?							
<i>Monoallelic variants identified</i>												
P.114	NFKB1	CVID	NM_003998.4	c.[1012_1012delT]; [1012=]	p.([Ser338Leufs*94]); ([Ser338=])			35	NA	NA	19, 32	31
P.183	NFKB1	CVID	NM_003998.4	c.[1012_1012delT]; [1012=]	p.([Ser338Leufs*94]); ([Ser338=])						19, 32	
P.122	NFKB1	CVID	NM_003998.4	c.[904dupT]; [904 =]	p.([p.Ser302Phefs*7]); ([Ser302])	RS773694113	0.000008287	34	NA	NA	19, 32	
P.150	NFKB1	CVID	NM_003998.4	c.[1726dupA]; [1726]	p.([Ile576Asnfs*6]); ([Ile576=])			35	NA	NA	19, 32	
P.200	NFKB1	CID	NM_003998.4	c.[875_875delG]; [875=]	p.([Gly292Valfs*140]); ([Gly292=])						19, 32	
P.210	NFKB1	CVID	NM_003998.4	c.[470G>C]; [470=]	p.([Arg157Pro]); ([Arg157=])			33	Probably damaging	D	19, 32	
P.232	NFKB1	CVID	NM_003998.4	c.[691C>T]; [691=]	p.([p.Arg231Cys]); ([Arg231=])		0.00004949	24	Possibly damaging	T		
P.233	NFKB1	CVID	NM_003998.4	c.[691C>T]; [691=]	p.([Arg231Cys]); ([Arg231=])		0.00004949					
P.254	NFKB1	Unclassified antibody deficiency	LRG_1316t1	c.[731-3C>G]; [731=]	LRG_1316p1:p.(=)			17	NA	NA		
P.26	NFKB2	CVID	NM_001077494.3	c.[1097G>A]; [1097=]	p.([Gly366Asp]); ([Gly366=])		0.00002845	19	Probably damaging	T		8, 33
P.181	NFKB2	CVID	NM_001077494.3	c.[2557C>T]; [2557=]	p.([Arg853*]); ([Arg853])	RS397514332	0.000005329	38	NA	T	33	
P.236	NFKB1A	CID	NM_020529.3	c.[106T>G]; [106=]	p.([Ser36Ala]); ([Ser36=])			25	Probably damaging	T	34	35
P.154	STAT3	CVID	NM_003150.4	c.[653T>C]; [653=]	p.([Val218Ala]); ([Val218=])			27	Benign	T	36	37

Table 2 (continued)

PatiID	Gene	Diagnosis	Transcript ID	Transcript variant	Protein variant	dbSNP-ID	gnomAD frequency	CADD score	PolyPhen2_ Pred	SIFT_ Pred	Variant ^a Ref.	Gene ^b Ref.
P.278	STAT3	CID	NM_003150.4	c.[1276T>C]; [1276=]	p.([Cys426Arg]); ([Cys426=])			22	Benign	T	38	
P.282	STAT3	CVID	NM_003150.4	c.[859T>G]; [859=]	p.([Leu287Val]); ([Leu287=])			20	Probably damaging	D	39	
P.265	STAT3	CID	NM_003150.4	c.[0.2144C>T]; [2144=]	p.([Thr715Met]); ([Thr715=])			21	Possibly damaging	T	39–40	
P.227	STAT3	Unclassified antibody deficiency	NM_003150.4	c.[0.2144C>T]; [2144=]	p.([Thr715Met]); ([Thr715=])			21	Possibly damaging	T	40	
P.246	STAT3	HIES	NM_003150.4	c.[0.1907C>T]; [1907=]	p.([Ser636Phe]); ([Ser636=])			24	NA	NA	12	
P.56	CTLA4	CVID	NM_005214.5	c.[2T>C]; [2=]	p.([?]); [Met1])			24	Possibly damaging	D	41	41
P.165	CTLA4	CID	NM_005214.5	c.[118G>A]; [118=]	p.([Val40Met]); ([Val40=])			25	Probably damaging	D		
P.215	CTLA4	CVID	NM_005214.5	c.[118G>A]; [118=]	p.([Val40Met]); ([Val40=])			25	Probably damaging	D		
P.263	IRF2BP2	CVID	NM_001077397.1	c.[1597T>G]; [1597=]	p.([Cys533Gly]); ([Cys533=])			24	Probably damaging	D		8, 42
P.159	IRF2BP2	CVID	NM_001077397.1	c.[1597T>G]; [1597=]	p.([Cys533Gly]); ([Cys533=])			24	Probably damaging	D		
P.257	RELA	CVID	NM_001145138.2	c.[622G>A]; [622=]	p.([Glu208Lys]); ([Glu208=])			34	Probably damaging	D		43
P.257	GATA2	CVID	NM_001145662.1	c.[1343C>A]; [1343=]	p.([Ser448Tyr]); ([Ser448=])			25	Possibly damaging	D		44
P.101	STAT1	CID	NM_007315.4	c.[1198C>G]; [1198=]	p.([Leu400Val]); ([Leu400=])			27	Probably damaging	D		45, 14
P.262	STAT1	CID	NM_007315.4	c.[800C>T]; [800=]	p.([Ala267Val]); ([Ala267=])			22	NA	NA	45	
P.164	TCF3	CVID	NM_001136139.4	c.[1604T>C]; [1604=]	p.([Leu535Pro]); ([Leu535=])	RS373228083	0.000008077	14,5	Probably damaging	D		46
P.166	TCF3	CVID	NM_001136139.4	c.[1660C>T]; [1660=]	p.([Arg554Trp]); ([Arg554=])			17	Probably damaging	D		
P.270	MYH9	CVID	NM_002473.6	c.[3838G>A]; [3838=]	p.([Val1280Met]); ([Val1280=])	RS141582478	0.000006759	23	Possibly damaging	D		47–48
P.253	VAV1	CVID	NM_001258206.2	c.[29G>T]; [29=]	p.([Trp10Leu]); ([Trp10=])			29	Probably damaging	D		8
P.253	VAV2	CVID	NM_003371.4	c.[2327C>A]; [2327=]	p.([Pro776His]); ([Pro776=])			27	Probably damaging	D		
P.294	GATA2	Unclassified antibody deficiency	NM_001145662.1	c.[481C>G]; [481=]	p.([Pro161Ala]); ([Pro161=])	RS34799090	0.008156	26	Probably damaging	D		
P.267	SEC61A2	CID	NM_001142628.1	c.[718C>T]; [718=]	p.([Arg240Cys]); ([Arg240=])		0.000008239	29	Probably damaging	D		
P.63	PIK3R1	CVID	NC_000005.9	c.[635-11T>A]; [635=]	p.([=])		0.000008434	16	NA	NA		49
P.63	GATA2	CVID	NM_001145662.1	c.[30G>C]; [30=]	p.([Trp10Cys]); ([Trp10=])			27	Possibly damaging	D		

Table 2 (continued)

PatiID	Gene	Diagnosis	Transcript ID	Transcript variant	Protein variant	dbSNP-ID	gnomAD frequency	CADD score	PolyPhen2_ Pred	SIFT_ Pred	Variant ^a Ref.	Gene ^b Ref.
P.127	PIK3R4	Unclassified antibody deficiency	NM_014602.3	c.[1039C>T]; [1039=]	p.([Arg347Trp]); ([Arg347])		0.0013	32	Possibly damaging	T		
P.124	SOC51	CVID	NM_003745.2	c.[147_153del]; [147=]	p.([Pro50Thrfs*33]); ([Pro50=])		0.0001096	14	NA	NA		50
P.135	PIK3R1	CVID	NM_181504.4	c.[18G>T]; [18=]	p.([Trp6Cys]); ([Trp6=])		0.00023	16	Probably damaging	D		49
P.182	PIK3R4	CVID	NM_014602.3	c.[1039C>T]; [1039=]	p.([Arg347Trp]); ([Arg347])		0.001376	32	Possibly damaging	T		
P.264	REL	Unclassified antibody deficiency	NM_001291746.2	c.[395T>C]; [395=]	p.([Val132Ala]); ([Val132=])			24	Probably damaging	NA		43
P.266	PIK3R4	CVID	NM_014602.3	c.[3341A>G]; [3341=]	p.([Tyr1114Cys]); ([Tyr1114=])	RS372167716	0.000048	28	Probably damaging	T		
<i>X-linked variants</i>												
P.279	BTk	Agammaglobulinemia	NM_000061.3	c.[427C>T]; [0]	p.His143Tyr			26	Probably damaging	NA		51
<i>Compound heterozygous variants</i>												
P.64	RAG1	CID	NM_000448.3	c.[1431del; 1123C>G]; [1420C>T]	p.([Phe478Serfs*14; His375Asp]); ([Arg474Cys])			NA, 25,29	Probably damaging	NA, D	52–53	54
<i>Uncertain allele phase</i>												
P.108	USP8	CVID	NM_001128610.3	c.1888A>T (i)1888_1890del	p.([Lys630*](i) (Lys630del)			41	NA	T		55
P.182	RELB	CVID	NM_006509.4	c.1291G>C (i) 1579G>A	p.([Glu431Gln](i) (Glu527Lys)			31, 17	Possibly damaging	T		56

CADD, combined annotation-dependent depletion; CID, combined immunodeficiency; CVID, common variable immunodeficiency; D, deleterious; F, female; freq, frequency; gnomAD, genome aggregation database; M, male; MSC, mutation significance cut-off; NA, not available; Pat. ID, patient identification number; pred., prediction; ref., reference; ref. seq., reference sequence; SCID, severe combined immunodeficiency; T, tolerated; tNGS, targeted next-generation sequencing. ^aPrevious report on the pathogenicity of the identified variant. ^bPrevious report on the involvement of the mutated gene in primary immunodeficiency.

Fig. 2. Summary of genes whose predicted disease-causing variations were detected in a cohort of PID patients, who mostly had sporadic immunodeficiency, falling under a PAD. PAD, primary antibody deficiency; PID, primary immunodeficiency.

tors such as consanguinity, family history of PID, age at diagnosis of PID, the clinical diagnosis of PID, and its clinical manifestations on the diagnostic rate of tNGS. As expected, consanguinity or familial history of PID led to a considerably higher diagnostic rate (Table 3). In particular, in the subgroup of patients with consanguinity or a family history of PID (37/294), the diagnostic performance of tNGS would be 48.6% (18/37). In contrast, excluding those patients would result in reduction of the diagnostic yield to 10.5% (27/257). Regarding the clinical diagnosis of PID, the highest diagnostic rate has been identified in the subgroup of patients with CID. Among evaluated clinical manifestations, a predicted disease-causing variant was more commonly found among the subgroup of patients with enteropathy and organ-specific autoimmunity.

Therapeutic Consequences of Genetic Diagnosis through tNGS

Genetic testing through tNGS had a higher diagnostic rate among patients with immune dysregulation, manifesting as organ-specific autoimmunity or enteropathy,

identifying among else, monoallelic gain-of-function variants in *STAT1* and *STAT3*, *CTLA4* variants leading to *CTLA4* insufficiency, *PIK3R1* variants, likely resulting in hyperactivation of *PI3Kδ*, and biallelic *LRBA* variants, likely causing *LRBA* deficiency. As discussed above, targeted therapies can be considered for those genetic diagnoses, especially for treating manifestations of immune dysregulation, such as autoimmune and/or lymphoproliferative disease.

Based on genetic diagnosis, already 2 patients diagnosed with *STAT3* gain-of-function are treated with baricitinib, due to their progressive interstitial lung disease (P.227, P.265). A paediatric patient harbouring a *STAT3* gain-of-function variant (P.278) developed severe ILD, which led to aggressive immunosuppressive treatment with glucocorticosteroids and hydroxychloroquine mycophenolate mofetil, intravenous immunoglobulins, and etanercept, which were all ineffective in controlling its progressive course. Consequently, this child underwent bilateral lung transplantation at the age of 14 years and died 12 months later due to treatment-resistant chronic allograft dysfunction. The genetic analysis by tNGS has been only performed post-mortem and revealed a mutation in *STAT3* (c.1276T>C, p.C426R), whose gain-of-function effect has been confirmed [38]. Timely genetic diagnosis, in that case, would have led to employing treatment with a JAK-inhibitor or tocilizumab, which appears more effective in treating immune dysregulation in the context of *STAT3* gain-of-function syndrome. Similar to the *STAT3* gain-of-function patients, a patient with *STAT1* gain-of-function (P.101) displayed steroid-refractory autoimmune haemolytic anaemia and lymphopenia, which were successfully controlled after introducing treatment with baricitinib. Among identified patients with a *CTLA4* mutation, 2 displayed a therapy-refractory enteropathy (P.165, P.215) and will be offered an abatacept treatment.

Besides targeted treatment of immune dysregulation, genetic diagnosis may lead to timely haematopoietic stem cell transplantation (HSCT), especially in patients with CIDs. Among tested patients, we identified a patient with *RAG1* deficiency (P.64). This patient was diagnosed with progressive multifocal leukoencephalopathy, which had a lethal outcome. Considering the link between *RAG1* deficiency and SCID or CID [52, 53], earlier genetic diagnosis in that patient could have led to a timely consideration of HSCT. Similar is the case of the patient diagnosed with *IκBα* gain-of-function, who currently suffers from recurrent infections, including pneumonia, and already has severe bilateral bronchiectasis (P.236) [34]. Given the lethal outcome of this IEL, timely genetic diagnosis at the age of

Table 3. Factors associating with the identification of predicted disease-causing genetic variants

Variable	Predicted pathogenic variant (N = 45)	No pathogenic variant (N = 249)	OR (95% CI)	p value ⁺
Familial history of PID	16	18	7.08 (3.26–15.39)	<0.0001***
Consanguinity	5 ¹	2	5.44 (2.90–82.33)	0.0011**
Age group at diagnosis ²				
0–4 years	7	7	5.9 (1.96–17.76)	0.0028**
5–18 years	14	48	1.72 (0.85–3.49)	0.17 (ns)
>18 years	23	177	0.32 (0.17–0.62)	0.0009***
CVID	25	202	0.29 (0.15–0.57)	0.0004***
CID	13	16	5.92 (2.61–13.43)	<0.0001***
Unclassified antibody deficiency	4	17	1.33 (0.43–4.16)	0.54 (ns)
Infections only disease	14	94	0.74 (0.38–1.47)	0.5 (ns)
Lymphadenopathy	8	36	1.28 (0.55–2.97)	0.65 (ns)
Splenomegaly	10	64	0.83 (0.39–1.76)	0.71 (ns)
Enteropathy	10	17	3.90 (1.65–9.20)	0.003**
ILD	4	16	1.42 (0.45–4.47)	0.52 (ns)
Organ-specific autoimmunity	20	38	4.86 (2.46–9.61)	<0.0001***
Autoimmune cytopenia ³	9	35	1.85 (0.86–3.98)	0.13 (ns)
Atopic disease ⁴	9	39	1.35 (0.60–3.02)	0.51 (ns)
Granulomatous disease	3	22	0.74 (0.21–2.57)	0.78 (ns)
Malignancy	4	24	0.92 (0.30–2.78)	1 (ns)

CI, confidence interval; CID, combined immunodeficiency; CVID, common variable immunodeficiency; ILD, interstitial lung disease; ns, not significant; OR, odds ratio; PID, primary immunodeficiency disorder; SPAD, specific antibody deficiency. ⁺ $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. ¹ Including 3 patients from the same family. ² Analysis based on 276/294 studied patients with the known year of diagnosis. ^{3A} Autoimmune hemolytic anemia (AIHA) and/or immune thrombocytopenic purpura (ITP). ^{4A} Atopic dermatitis and/or allergic rhinitis and/or asthma.

the first diagnosis might have led to HSCT before the currently existing severe lung damage.

In summary, in 4/294 (1.4%) patients, genetic diagnosis led to the introduction of a targeted therapy, tailored to revert the consequences of the identified genetic defect. However, considering patients with retrospective, post-mortem diagnosis, or currently treatment-refractory immune dysregulation, the fact that some of the identified predicted pathogenic variants, such as those in *LRBA* or *PIK3R1*, have not been functionally validated, genetic diagnosis through tNGS could affect the follow-up and/or result in therapeutic consequence in substantially higher number of tested patients.

Discussion/Conclusion

tNGS has been employed in the present study to investigate the genetic basis of immunodeficiency in a cohort of patients who mainly had sporadic PID, falling under a PAD. This sequencing approach revealed a likely pathogenic genetic variant in approximately 15% of tested pa-

tients. This diagnostic rate was relatively low, though similar to the one reported in some previous genetic studies, employing WES or WGS on cohorts of patients with sporadic immunodeficiency [9, 50, 59]. The diagnostic rate of tNGS was significantly higher in the subgroup of patients with a familial history of PID or those with an early onset of disease. CID or immunodeficiency complicated with enteropathy or organ-specific autoimmunity were additional factors associating with a significantly higher diagnostic rate. The overall low diagnostic rate of tNGS in the present study may be explained through the dependence of the diagnostic performance of genetic testing and tNGS on factors such as consanguinity or family history with PID [60], which were present in a minority of tested patients. Further, the limited diagnostic yield of tNGS may reflect the polygenic origin of PID and the pathogenic role of additional yet unidentified genetic and/or epigenetic modifiers [3]. Given the increasing number of genes linked to IEI, additional disease-causing genes may be identified in the near future. For this reason and as the gene panel tested in the present study did not include all currently known PID genes, employing a

broader sequencing approach, such as WES, may have resulted in a higher diagnostic rate.

Overall, 3.1% of tested PID patients harboured a predicted pathogenic *NFKB1* variant, which was the most common genetic defect among tested patients. This finding is in line with previous studies [61, 62], which identified heterozygous loss-of-function variants in *NFKB1* as the most common genetic defect in patients with CVID. In compliance with previous studies of large cohorts of PID patients with *NFKB1* mutations, this subset of patients displayed variable immunodeficiency, ranging from infections-only antibody deficiency to CID and marked immune dysregulation [19]. Characterization of phenotypes of patients with immunodeficiency linked to heterozygous *NFKB1* mutations revealed a relatively high prevalence of autoimmune and lymphoproliferative manifestations [19, 32, 61]. Together with the recent identification of *NFKB1* mutations in patients with sheer rheumatic disease and secondary hypogammaglobulinaemia [63], the latter leads to the assumption that NF- κ B1-related disease is primarily a condition of immune dysregulation rather than mere immunodeficiency [32]. The observation of progressive hypogammaglobulinaemia in currently asymptomatic mutation carriers and the likely concurrent progressive course of immunodeficiency [61] may lead to a later disease onset in some currently asymptomatic mutation carriers; immunodeficiency might be precipitated from the introduction of immunomodulatory regimens for their autoimmune manifestations [63]. The aforementioned finding emphasizes the importance of early molecular diagnosis of NF- κ B1-related disease and the need to develop targeted NF- κ B pathway-based therapeutic intervention.

The increasing availability of sequencing technologies, especially of NGS, has improved our understanding of the pathogenesis of PID and paved the way for targeted therapeutic intervention with agents directed at reverting the disease-causing molecular abnormality or its pathophysiological consequences [64, 65]. Therefore, such targeted therapies and identifying the genetic basis of PID can be essential for patients with manifest immune dysregulation as conventional immunomodulatory regimens may exert an immunosuppressive effect, aggravating their immunodeficiency or may only inadequately control autoimmune or lymphoproliferative manifestations. Already employed individualized therapeutic approaches in the field of IEI include mTOR- or PI3K δ -specific inhibitors in patients with mutations in *PIK3CD*, *PTEN*, or *PIK3R1* causing activated PI3K δ syndrome [29, 49]; abatacept (CTLA4-Ig) in patients with LRBA deficiency; CTLA4 insufficiency or DEF6 deficiency [16, 17, 66]; jakinibs in patient with

STAT1 or STAT3 gain-of-function [14]; and the CXCR4 antagonist plerixafor in patients with warts, hypogammaglobulinaemia, immunodeficiency, and myelokathexis syndrome [67]. Despite the lack of randomized controlled clinical trials, evidence from case reports and case series suggests both the excellent tolerability and efficacy of such targeted therapies. Together with their focused biological effect, it makes them attractive, especially when compared with conventional immunomodulatory regimens. Here, molecular diagnosis through tNGS resulted in treatment with baricitinib in a subgroup of patients with gain-of-function mutations in *STAT1* or *STAT3*. Further, post-mortem identification of therapeutically relevant mutations in 3 studied patients, who directly or indirectly died of treatment-refractory immune dysregulation, highlights the importance of early genetic diagnosis, especially in patients with the autoimmune or lymphoproliferative disease as this would have resulted in timely consideration of targeted therapy. In the current study, the rate of patients who received targeted therapies based on genetic diagnosis was low and may underestimate the clinical significance of genetic diagnosis. The main reasons for the lack of a therapeutic consequence were delayed genetic testing, including cases with post-mortem diagnosis, and the lack of functional validation of some of the identified variants. Further, genetic diagnosis should lead to genetic counseling of family members and family cascade testing, which can lead to genetic diagnosis in additional patients and may expand the clinical consequences of genetic testing.

Generally, in comparison to whole-exome (WES) or whole-genome sequencing (WGS), the targeted sequencing gene panel has an increased sequencing depth and allows rapid, cost-effective, and simultaneous sequencing of multiplexed samples in 1 sequencing run [68]. However, given the growing number of genes linked to IEI, it may require >1 gene panel for the genetic work-up PID. Despite testing for variations in genes most commonly linked to IEI or currently considered therapeutically relevant, the latter limitation of tNGS may underestimate the prevalence of monogenic disease in the studied cohort as discussed above. For this purpose and based on a recommendation of the IUIS experts committee, the best way to identify a disease-associated genetic variation in a new PID patient might consist of a tNGS followed by WES or WGS for unresolved cases [43]. However, given the currently dropping prices of WES and WGS, this suggestion may need to be revised. An additional limitation of our study is the *in silico* evaluation of the pathogenicity of identified variants, which may overestimate the prevalence of PID-related variants.

Even in the case of a definite molecular diagnosis, for most patients harbouring an IEI-causing mutation, the disease does not follow Mendelian inheritance. The identified pathogenic variant is commonly associated with incomplete penetrance variable expressivity, rendering disease course and therapeutic choice difficult. Therefore, precision medicine, whose scope is to be served through routine genetic testing, may be further facilitated by identifying an additional germline or somatic genetic modification as well as epigenetic factors and identifying the role of environmental factors [69–71]. A better understanding of those factors could provide additional insight into disease pathogenesis's fundamental processes, offering a better perspective towards genetic counselling and precision medicine.

In conclusion, the employed Haloplex tNGS-approach is a time- and cost-effective sequencing method, providing a unique diagnostic tool for identifying known or novel variants in PID-associated genes. This approach had a high diagnostic rate, especially within the subgroup of patients with CID or immune dysregulation, for whom genetic diagnosis may affect therapeutic decision-making. However, given the increasing number of genes associated with IEI, which at the moment amount to >430, a panel-sequencing approach is ineffective in integrating all disease-relevant genetic loci, highlighting the importance of sequential sequencing, where negative-targeted NGS may be followed by WES or WGS. The latter may be especially relevant for patients who, in addition to antibody deficiency, display cellular immunodeficiency or those with complications such as autoimmunity, lymphoproliferation, or malignant disease as identifying the genetic cause of disease may affect follow-up procedures or therapeutic decision-making.

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Statement of Ethics

The institutional medical Ethical Committee at the Hannover Medical School approved the study (ethics approval number: Nr.8875_BO_K_2020). Written consent of all study participants or their parents or guardians was obtained. The authors have no ethical conflicts to disclose.

Conflict of Interest Statement

The authors have no other relevant affiliations or financial participation with any organization or entity with financial interest.

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Author Contributions

Research design was conceived by F.A., G.S., B.G., and R. E. S.; sample collection and analyses were performed by F. A., N. D., G. S., and U.B.; data analysis was performed by F.A. and G.S.; writing and contributing to writing of the manuscript was performed by F. A., G.S., and all the authors.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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