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ORIGINAL ARTICLE

Clinical efficacy of a next-generation sequencing gene panel for primary immunodeficiency diagnostics

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Primary immunodeficiencies (PIDs) are rare monogenic inborn errors of immunity that result in impairment of functions of the human immune system. PIDs have a broad phenotype with increased morbidity and mortality, and treatment choices are often complex. With increased accessibility of next-generation sequencing (NGS), the rate of discovery of genetic causes for PID has increased exponentially. Identification of an underlying monogenic diagnosis provides important clinical benefits for patients with the potential to alter treatments, facilitate genetic counselling, and pre-implantation diagnostics. We investigated a NGS PID panel of 242 genes within clinical care across a range of PID phenotypes. We also evaluated *Phenomizer* to predict causal genes from human phenotype ontology (HPO) terms. Twenty-seven participants were recruited, and a total of 15 reportable variants were identified in 48% (13/27) of the participants. The panel results had implications for treatment in 37% (10/27) of participants. *Phenomizer* identified the genes harbouring variants from HPO terms in 33% (9/27) of participants. This study shows the clinical efficacy that genetic testing has in the care of PID. However, it also highlights some of the disadvantages of gene panels in the rapidly moving field of PID genomics and current challenges in HPO term assignment for PID.

KEYWORDS

clinical diagnostics, human phenotype ontology, next-generation sequencing, primary immunodeficiency

1 | INTRODUCTION

Primary immunodeficiency (PIDs) are inborn errors of immunity that encompass a collection of rare monogenic diseases resulting in impairment of 1 or more functions of the human immune system. PID has a broad phenotype including severe susceptibility to infections, allergy, autoimmunity/inflammation, and malignancy.[1] These disease manifestations cause increased morbidity and mortality in patients, and treatment choices are often complex. With the increased accessibility of next-generation sequencing (NGS), the rate of discovery of genetic causes for PID has increased exponentially in recent years, with now over 300 monogenic causes for PID described.[2,3]

The increasing number of monogenic causes identified for PID, coupled with knowledge of how these genetic alterations impact on protein and cellular functions, has led to the introduction of precision medical interventions targeting specific defects in patients.[4,5] Traditional treatments for PID consist of prophylaxis against infection, with antimicrobials and immunoglobulin, iatrogenic immunomodulation with high inherent risk associated with additional untargeted immunosuppression, and bone marrow transplantation in severe cases. Precision therapies now include targeted immunosuppression in cases of autoimmune/inflammatory manifestations and gene therapy to correct germline errors in autologous haematopoietic stem cells.[6,7] These therapeutic approaches have the potential to dramatically improve the prognoses for patients with PID.[7–9] Precision

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treatments, as well with genetic family counselling and pre-implantation diagnostics, are only possible with knowledge of the causal monogenic variant in patients, and recent progressions in diagnostics and treatments underline the importance of genomic investigations in the clinical care of patients with PID.[10,11]

Current genomic methodologies used for the investigation vary from Sanger sequencing of single candidate genes, to NGS gene panels, whole exome sequencing (WES) and now whole genome sequencing (WGS).[12–14] The most widely used technique within routine clinical care remains NGS gene panels due to economic, data handling, and the result turnaround time advantages.[15] NGS gene panels can provide a comprehensive method for diagnostics in PID, with reported diagnostic rates between 15% and 70%, depending on the PID population and phenotypic criteria of patients assessed.[16–19]

Due to the importance that a genetic diagnosis can have in the clinical management of PID, we investigated the diagnostic utility of an NGS panel of 242 PID genes in clinical practice. To reflect patients encountered within routine clinics, we recruited and collected phenotypic data from a heterogeneous range of PID participants using human phenotype ontology (HPO) terms.[1,20] We also assessed the ability of *Phenomizer* to construct candidate gene lists from participant HPO terms.[21]

2 | MATERIALS AND METHODS

2.1 | Participants

Twenty seven genetically undiagnosed participants with a phenotype compatible with PID[1] and a diagnosis of PID according to the European Society for Immunodeficiencies *(ESID)* (https://esid.org/Working-Parties/Registry/Diagnosis-criteria) were enrolled from a single centre. Written informed consent was obtained from all participants (REC reference 12/NW/0794, ERGO 21562).

2.2 | Study design

This study was a prospective cohort study. Inclusion criteria included a clinical diagnosis of PID, attendance at a Primary Immunodeficiency Clinic (Adult or Paediatric) at University Hospital Southampton NHSFT and the ability of the participant or parent/guardian to provide written informed consent. Exclusion criteria included prior knowledge of the genetic cause of PID and/or previous bone marrow transplant or gene therapy. A target cohort size of 20-30 participants was chosen based on the results of previously published studies.[19,22]

2.3 | Clinical phenotyping

Clinical phenotyping was performed from analysis of patient medical records and laboratory results. Phenotypic data were recorded in HPO terms (Table S1, Supporting Information).

2.4 | DNA preparation

Five millilitres of EDTA blood was taken from participants. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, DE) according to the manufacturer's instructions. DNA quality was checked by Nanodrop spectrophotometry (Thermofisher, Massachusetts). DNA quality requirements were a 260/280 ratio of 1.70-1.90 and a minimum DNA concentration of 20 ng/μL.

2.5 | Library preparation and sequencing

DNA libraries were prepared using a Nextera Rapid Capture Enrichment protocol according to the manufacturer's instructions (TruSight One Sequencing Panel, FC-141-1007, Illumina, California). Briefly, individual genomic DNA samples were fluorometrically quantified and 50 ng of DNA was enzymatically fragmented and tagged. Resulting genomic libraries were then dual indexed and amplified by polymerase chain reaction (PCR) using sample-specific index primer pairs. Five hundred nanograms of each indexed library was pooled on a 12 samples per protocol basis. The prepared pools were subjected to 2 rounds of library enrichment, biotinylated capture probes were hybridised to the DNA, captured with streptavidin-coated magnetic beads, stringency washed and eluted to produce the enriched DNA library. The enriched DNA library was then further amplified by PCR. Three amplified enriched libraries were equimolar pooled to produce a final library for sequencing containing 36 individual samples. Sample purification steps were performed throughout the procedure as recommended. Prepared libraries were denatured and diluted to 1.8 pmol as per the manufacturer's instructions (15048776, Illumina, California). A 2× 151 bp paired end sequencing was performed using a NextSeq 500 High Output v2 Kit (FC-404-2004, Illumina, California) on a NextSeq 500 sequencing system (SY-415-1001, Illumina, California) according to the manufacturer's instructions.

2.6 | Bioinformatic analysis

Per-cycle BCL basecall files were converted into demultiplexed per-read FASTQ files using bcl2fastq software (Illumina, California). Resulting FASTQ files were quality checked and processed according to GATK best practice guidelines and aligned to the GRCh37/hg19 reference genome using BWA-MEM. Variants were called from the Binary Alignment Map (BAM) file using GATK: unified genotype caller. BAM and Variant Call Format (VCF) files were then uploaded and annotated by the online module Sapientia (Congenica, Cambridge, UK) for filtering and analysis of variants.

2.7 | Variant filtering

Data from the TruSight One (Illumina, California) 4813 gene exon panel were filtered to include only the virtual PID gene panel of 242 genes (Table S2). Variants were then filtered to only include missense, frameshift, nonsense, and canonical splice site variants, single-nucleotide polymorphisms (SNPs) and indels. Remaining variants were then filtered to a minor allele frequency (MAF) of <0.01 in the Exome Aggregation Consortium (ExAC).[23] Variants were then interrogated using in silico predictive tools PolyPhen2,[24] SIFT,[25] and Ensembl variant effector predictor (VEP).[26] BAM file read alignments were visually inspected for each variant identified using Integrative Genomics Viewer (IGV).[27] Variants were subsequently graded according to the

American College of Medical Genetics (ACMG) criteria.[28,29] Reportable variants were defined as those classed by ACMG criteria as pathogenic, likely pathogenic, or variants with clinical implications for PID disease management through clinical risk modification in PID.

To investigate whether the finding of ≥2 reportable variants in any single individual presented distinct blended phenotypes or potential digenic/multigenic inheritance as the basis for disease, GeneMANIA was used to assess for interactions and expression of genes and proteins within cellular pathways.[30,31]

2.8 | *Phenomizer* candidate gene list filtering

HPO terms for each participant were input into *Phenomizer* (http://compbio.charite.de/phenomizer/) to generate gene lists. Candidate gene lists were then compiled for each individual (Table S4) using a cut-off of $P < .05$ for gene likelihood probabilities as calculated by Monte-Carlo random sampling with Bonferroni correction (a method known as Ontology Similarity Search with *P* values).[21]

3 | RESULTS

3.1 | Clinical phenotypes

All 27 participants had a range of phenotypes compatible with PID.[1] The number of HPO terms assigned to participants ranged from minimum of 2 to maximum of 13 (median = 6) (Figure 1 and Table S1).

3.2 | 242 PID gene panel results

There was coverage of >1× read depth in 99.52%, >30× read depth in 96.19%, and >50× read depth in 87.57% of the targeted exonic sequence across the 242 genes in the PID virtual panel (Table S2). The mean read depth over the reportable variants was 98 reads (Table 1). Sixty seven known PID genes are not covered by TruSight One Sequencing Panel and not included in the 242 PID gene panel (Table S3).

Forty-six percentage (13/27) of individuals investigated by the NGS PID gene panel had a monogenic diagnosis identified. Fifteen reportable variants were identified that were either graded as pathogenic/likely pathogenic and/or had clinical implications in the 13 participants. A variant in *TNFRSF13B*, despite previously being reported as pathogenic, was only determined as a variant of unknown significance according to ACMG criteria (Table 1). Novel variants were found in *NFKB1*, *IKBKG*, *GATA2*, *TAZ*, *PIK3CD* and *CTLA4*. Variants previously reported as pathogenic were identified in *PIK3CD*,[34] *IFNGR1*,[35] *STXBP2*,[38] *STAT1*,[37] *NLRP3*,[39] and *TNFRSF13B*.[40]

Seventy-three percentage (11/15) of reportable variants was loss-of-function and 28% (4/15) variants were gain-of-function (GOF). Variants were graded according to ACMG criteria with 47% (7/15) of identified variants being classified as pathogenic, 47% (7/15) classified as likely pathogenic, and 6% (1/15) a variant of undetermined significance (VUS), but in the clinical context considered reportable due to disease risk modification (Table 1).

P1 underwent an array comparative genomic hydridisation (aCGH) which identified a large 2 Mb monoallelic deletion within the 6q15 region (90511889-92156885). This deletion contained the genes *MDN1*, *BACH2*, *MAP3K7*, *CASP8AP2*, and *GJA10*. Haploinsufficiency of the transcription factor *BACH2* has recently been described to cause immunodeficiency with autoimmunity, which is a similar clinical phenotype to that of P1, making this the likely cause of the participant's PID.[41]

Two participants (P7 and P26) had 2 reportable variants identified. We assessed whether these 2 gene variants were likely to contribute to a blended phenotype of 2 distinct phenotypes that overlap within the individuals, or potentially contributed in a digenic mechanism for disease. Using GeneMANIA, we mapped the physical interactions of protein products, biological pathways, and genetic interactions of the genes *GATA2:NLRP3* and *STAT1:TNFRSF13B* which did not find evidence of interactions (Figure 2).

3.3 | *Phenomizer* candidate gene list results

Phenomizer-derived candidate gene lists identified the genes with reportable variants in 33% (9/27) of participants found to have a monogenic diagnosis (P2, P3, P5, P7, P16, P17, P23, P26, P27). *Phenomizer* did not identify the gene containing the reportable variant in

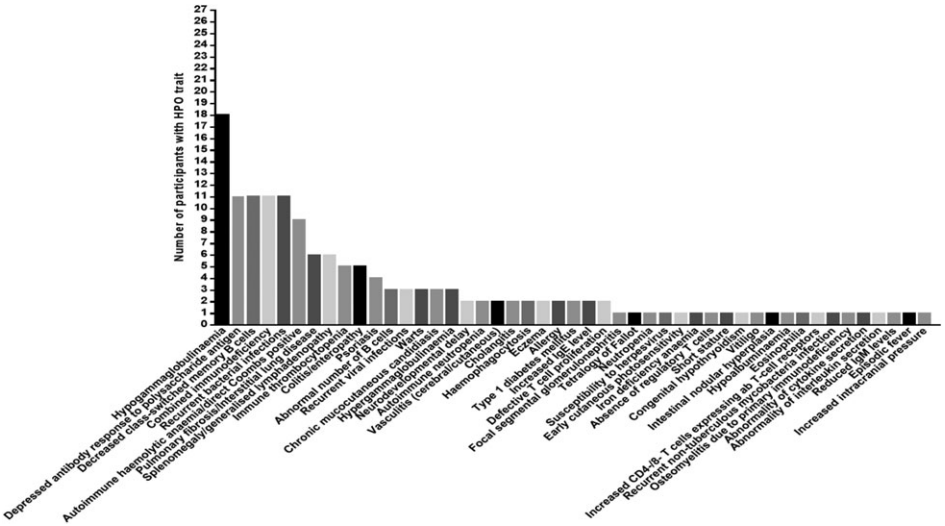


FIGURE 1 Number of human phenotype ontology terms assigned across the cohort displayed as a bar graph. Hypogammaglobulinaemia was the most frequently encountered being present in 66% (18/27) participants. There were a number of traits present only in single participants

TABLE 1 Reportable genetic results from participants with Online Mendelian Inheritance in Man (OMIM) database disease number, next-generation sequencing read depth over variant, in silico results, conservation scores (GERP)[32] and American College of Medical Genetics (ACMG) classification for participants with diagnoses from the primary immunodeficiency (PID) gene panel. PolyPhen2 scores: 0.0-0.15 benign; 0.15-0.85 possibly damaging; 0.85-1.0 probably damaging. SIFT scores: 0-0.05 damaging; 0.05-1.0 tolerated.[24,25] VUS = variant of undetermined significance

Participant	Genetic result	OMIM number	Read depth (split: Reference/ alternate)	ExAC MAF	PolyPhen2	SIFT	GERP	ACMG classification	Previous report of variant
P2	***NFKB1*** NM_003998 c.904dupT:p.Ser302Phefs*7	616576	103 (59/44)	0.000116	–	–	5.26	Pathogenic (Ia)PVS1, PM6, PP1-S, PM2	Novel
P3	***NFKB1*** NM_003998 c.904dupT:p.Ser302Phefs*7	616576	112 (51/61)	0.000116	–	–	5.26	Pathogenic (Ia)PVS1, PM6, PP1-S, PM2	Novel
P5	***IKBKG*** NM_003639 c.185G > A:p.Arg62Gln hemizygous	300291	92 (1/91)	<0.0001	0.971	0.01	5.6	Likely pathogenic (IV)PM2, PM5, PM6, PP2, PP3	Novel
P7	***GATA2*** NM_032638.4 c.526A > C:p.Thr176Pro	614172	71 (57/14)	0	0.979	0.03	4.01	Likely pathogenic (IV)PM2, PM6, PP3, PP1-S, PM2	Novel
	NLRP3 NM_004895.4 c.1469G > A:p.Arg490Lys	606416	161 (84/77)	0.00059	0.992	0.39	4.17	Likely pathogenic (II) PS1, PM6, PP2, PP3, PP5	Arostegui et al[39]
P10	***TAZ*** NM_181313 c.658A > G:p.Lys220Glu hemizygous	302060	158 (3/155)	0	0.94	0	5.33	Likely pathogenic (V)PM2, PM6, PP3, PP4	Novel
P16	***CTLA4*** NM_005214 c.118G > A:p.Val40Met	616100	118 (60/58)	0	0.595	0.02	5.28	Likely pathogenic (IV)PM2, PM5, PM6, PP2, PP3	Novel
P17	***PIK3CD*** NM_005026 c.3061G > A:p.Glu1021Lys	615513	67 (38/29)	0	0.997	0	3.72	Pathogenic (II)PS1, PS3, PS4, PM1, PM2, PM6	Lucas et al[33] Angulo et al[34]
P20	***CTLA4*** NM_005214 c.160G > A:p.Ala54Thr	616100	158 (84/74)	0	0.99	0	4.19	Likely pathogenic (II)PM2, PM6, PP2 PP3, PP4	Novel
P23	***IFNGR1*** NM_00041 c.819_822delTAAT:.p. Asn274Hisfs*2	615978	44 (22/22)	0	–	–	2.52	Pathogenic (Ia)PVs1, PS1, PM2, PS3, PP1-M, PM4	Jouanguy et al[35]
P24	***STXBP2*** NM_006949 c.1247-1G > C homozygous	613101	80 (2/78)	0.000676	–	–	4.08	Pathogenic (Ia)PVS1, PS1, PS3, PM2, PM3, PP3, PP4	Côte et al[38]
P25	***PIK3CD*** NM_005026 c.1213C > T:p.Arg405Cys	615513	71 (38/33)	0	0.982	0	5.35	Likely pathogenic (II)PS3, PM2, PP3, PP4	Rae et al[36] (this patient)
P26	***STAT1*** NM_007315.3 c.796G > A:p.Val266Ile	614162	74 (44/30)	0.00419	0.024	0.22	0.791	Pathogenic (II) PS3, PM2, PP3, PP4	Uzel et al [37]
	TNFRSF13B NM_012452 c.171G > C:p.Gln57His	240500	63 (27/36)	0.00090	0.966	0.02	2.19	VUS PS1, PP3	Almejún et al[40]
P27	***DOCK8*** NM _203447.3 c.4792G > T:p.Glu1598* Homozygous	243700	105 (2/103)	0	–	–	6.17	Pathogenic (Ia)PVS1, PS3, PM2, PM4	Novel

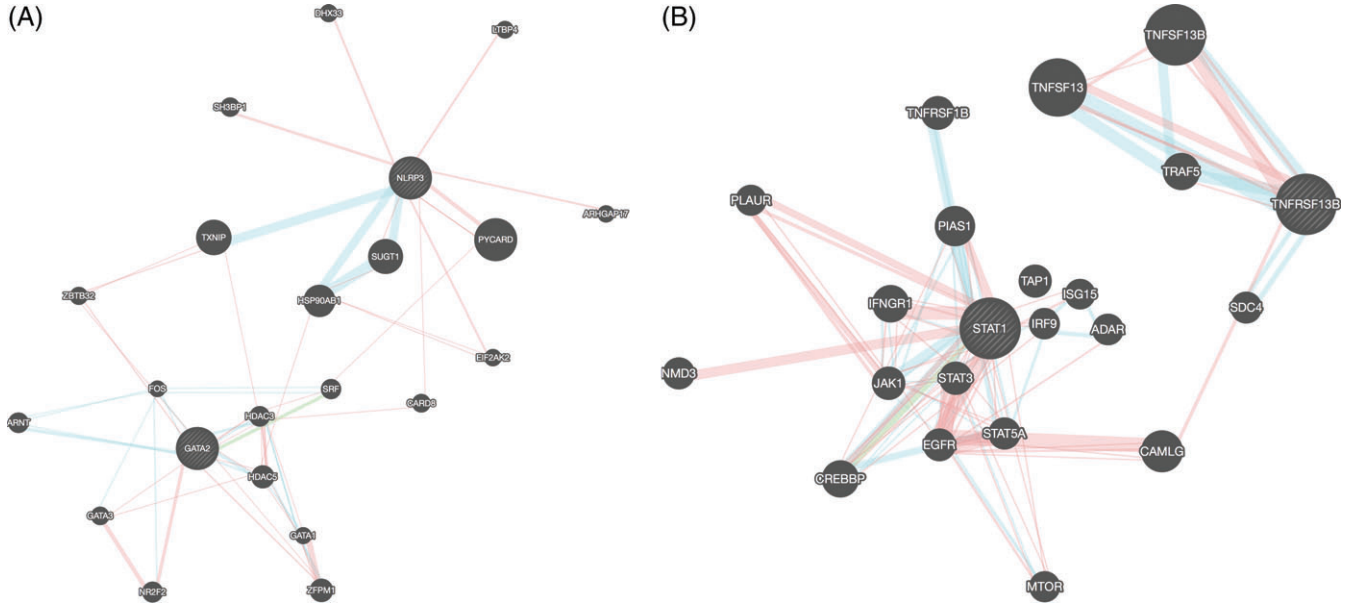


FIGURE 2 GeneMANIA maps showing the physical interaction (red), biological pathway (blue), and genetic (green) interactions of the 2 genes containing reportable variants from (A): P7; *GATA2* and *NLRP3* and (B): P26; *STAT1* and *TNFRSF13B*. Neither *GATA2* and *NLRP3* or *STAT1* and *TNFRSF13B* are directly connected by any biological pathway. These data suggest that these variants cause overlapping phenotypes of 2 discrete diseases, opposed to a digenic mechanism of disease through an additive genetic effect in vivo

37% (4/13) of participants (P10, P20, P24, P25). Candidate gene lists could not be generated for P9 and P11 due to none of the predicted genes returned by *Phenomizer* having a significance level of $P < .05$.

In those participants who remained undiagnosed (P1, P4, P6, P8, P9, P11, P12, P13, P14, P15, P18, P19, P21, P22), filtering of variants from the *Phenomizer* candidate gene lists did not identify any reportable variants (Table S4).

3.4 | Clinical management and treatment implications

The genetic information gained from the 242 PID gene panel had implications for clinical management and treatment in 37% (10/27) of the cohort (Table 2).

NFKB1 haploinsufficiency is described to cause a wide range of autoimmune/inflammatory diseases.[42,51] Both P2 and P3 presented with autoimmune cytopaenias and although both currently in remission, knowledge of the underlying genetic cause has meant that their immunosuppression with mycophenolate mofetil (MMF) should not be withdrawn due to high risk of disease relapse. Patients with *NFKB1* haploinsufficiency suffer with EBV reactivation and disease due to intrinsic natural killer cell defects.[52] From the clinical and immunological phenotypes of P2 and P3, we would not have expected this, but now with knowledge of the monogenic PID cause we will continually monitor EBV viral loads due to lymphoma risk.[43]

In P16 and P20, pathogenic *CTLA4* missense variants cause immunodeficiency with multi-system autoimmunity due to impaired regulatory T cell function.[45,53] A precision therapy can be administered in these patients with CTLA4:Ig fusion protein (Abatacept) to target autoimmunity.[44,45]

Activated phosphoinositide 3-kinase delta syndrome due to GOF variants in *PIK3CD* can cause lymphoproliferation and autoimmunity disease which is a significant cause of increased mortality.[54] These disease manifestations respond to inhibition of the hyperfunctional PIK3δ-AKT-mTOR pathway in lymphocytes of patients with use of mTOR inhibition by sirolimus or with PIK3δ inhibitors, such as idelalisib.[33,36]

Heterozygous frameshift variants in *IFNGR1* cause recurrent infection with low virulence non-tuberculous mycobacteria (NTM) due to expression of a non-functional cell surface receptor on patients' cells that acts as a "decoy receptor" for interferon-γ.[35] This inborn error against NTMs can be improved with use of exogenous interferon-γ to boost signalling through the residual receptors on effector cells.[35]

STAT1 GOF variants cause predisposition to chronic fungal infections as well as autoimmunity.[37,47] *TNFRSF13B* variants increase the risk of autoimmunity and granulomatous inflammation.[48] Knowledge of both these variants in P26 has improved pulmonary and autoimmune monitoring, allowing proactive rather than reactive medicine, and confidence in the need to continue iatrogenic immunosuppression in an immunodeficient individual. Precision medicine is available with the janus kinase inhibitor ruxolitinib for *STAT1* GOF patients.[46]

Biallelic pathogenic *DOCK8* variants cause a combined immunodeficiency that carries a high rate of mortality in early life.[55] As such, knowledge of this variant prompts early referral for bone marrow transplantation which improves outcome.[49,50]

The participants in whom a monogenic diagnosis was not established are undergoing broader diagnostic investigation such as WES/WGS, and functional interpretation of additional variants identified.

4 | DISCUSSION

This study highlights several advantages and disadvantages of NGS gene panels for diagnostic use in PID. The panel covered a relatively large number of PID genes at high read depth allowing a high sensitivity to detect SNP variants. This is an advantage of gene panels,

TABLE 2 Clinical management alterations/implications resulting from knowledge of the underlying genetic defect in 10/13 genetically diagnosed patients. MMF, mycophenolate mofetil drug

Participant	Genetic result	Clinical management/treatment implications	References
P2	***NFKB1*** NM_003998 c.904dupT:p.Ser302Phefs*7	Autoimmune surveillance, MMF EBV monitoring	Kaustio et al[42] Boztug et al[43]
P3	***NFKB1*** NM_003998 c.904dupT:p.Ser302Phefs*7	Autoimmune surveillance, MMF, EBV monitoring	Kaustio et al[42] Boztug et al[43]
P16	***CTLA4*** NM_005214 c.118G > A:p.Val40Met	Abatacept treatment	Lee et al[44] Schubert et al[45]
P17	***PIK3CD*** NM_005026 c.3061G > A:p.Glu1021Lys	Sirolimus, Idelalisib treatments	Lucas et al[33] Rae et al[36]
P20	***CTLA4*** NM_005214 c.160G > A:p.Ala54Thr	Abatacept treatment	Lee et al [44] Schubert et al[45]
P23	***IFNGR1*** NM_00041 c.819_822delTAAT:.p.Asn274Hisfs*2	Adjuvant interferon-gamma	Jouanguy et al[35]
P24	***STXBP2*** NM_006949 c.1247-1G > C homozygous	Herpes virus monitoring, aciclovir prophylaxis	Côte et al[38]
P25	***PIK3CD*** NM_005026 c.1213C > T:p.Arg405Cys	Sirolimus, Idelalisib treatments	Lucas et al [33] Lucas et al[33] Rae et al[36]
P26	***STAT1*** NM_007315.3 c.796G > A:p.Val266Ile ***TNFRSF13B*** NM_012452 c.171G > C:p.Gln57His	Autoimmune surveillance, MMF, ruxolitinib.	Weinacht et al[46] Toubiana et al[47] Salzer et al[48]
P27	***DOCK8*** NM _203447.3 c.4792G > T:p.Glu1598* Homozygous	Bone marrow transplant	Aydin et al[49] Al-Herz et al[50]

which have a smaller region of target DNA sequence than WES or WGS. In this study, a "virtual" PID gene panel was applied as a filter to the data generated from the Illumina TruSight One Sequencing Panel (which targets ~12 Mb of exonic DNA across 4813 genes in total). This represents a cost-effective way of generating sequence data limited to clinically relevant genes. By batching samples, we were able to produce results for £180/sample with a turnaround time of 4-8 weeks. In order to achieve this turnaround time. close working of a multi-disciplinary team comprising immunologists, technologists, bioinformaticians and geneticists was required working within the pipeline, and evaluating candidate variants in either a face-to-face or virtual multidisciplinary meetings. The mean read depth over reported PID gene variants in this series was 98 reads, which is more than is typically found in WES or WGS studies.[56] Owing to the smaller region of target DNA sequenced, gene panels generate less data than WES/WGS and therefore are faster to analyse with far fewer variants to interpret than would be expected through an untargeted approach. The risk of incidental/additional genetic findings can also be minimised using a rational, targeted approach to data analysis and interpretation. Together these factors represent a compelling argument for the use of gene panels (albeit as "virtual" panels applied to broader datasets) in the clinical diagnostic investigation of patients. In contrast, research-based analysis, demands wider scale unbiased genomic investigations such as WES/WGS.

In this study the gene panel had an encouraging diagnostic rate at 48% from a genetically undiagnosed phenotypically heterogeneous PID cohort. Knowledge of the monogenic cause for PID had implications for treatments in 37% (10/27) of the cohort, showing the clinical importance of genetic investigation in PID. These data show that this NGS gene panel approach is efficacious for the investigation of PID patients who are representative of those encountered in clinical practice. As such, this gene panel might reasonably be offered as a first-line genetic investigation for PID patients in the clinic who have a clinical diagnosis of PID as defined by the ESID criteria.

An inherent limitation of gene panels is lack of inclusion of recently described/novel genes. This 242 PID gene panel does not include 67 known PID genes described since its development (S3 Table). Many of these missing genes have subsequently been shown to be prevalent in cohorts of PID patients.[57] Another disadvantage is highlighted by the results of P1, in whom a monogenic diagnosis was not reached with the PID gene panel, but an aCGH identified a large structural variant including the *BACH2* gene as the cause of the patient's condition.[41] Large structural variants are not detectable from NGS gene panels, and although bioinformatics software exists to investigate for structural variants in NGS WES data it remains challenging due to the PCR amplification of DNA during library preparation and need for read de-duplication. This is an advantage of WGS which does not involve PCR amplification of DNA and has contiguous paired-end reads across the genome allowing for the detection of larger structural variants from read depths and split paired-end reads.[58,59]

In participants P7 and P26, 2 variants were identified that have been reported to be monogenic causes of PID (Table 1). Digenic mechanisms for inherited genetic diseases are well described, but have not been identified in the field of PID.[60] Two distinct monogenic causes for PID have been rarely reported in the same individual resulting in a blended phenotype of 2 underlying monogenic PIDs.[61] Results of large scale genomic investigation studies suggest that ~5% of individuals with rare disease, initially considered to have a single monogenic cause for disease, will have a blended phenotype due to

the presence of ≥2 penetrant monogenic variants.[31] Methods to determine whether in these cases the presence of these 2 reportable variants represent digenic inheritance models for a single disease entity (that would not manifest without both variants being present) or a blended phenotype due to dual monogenic diagnoses overlapping within the same individual have been developed.[31] Results of our cases show that neither *GATA2:NLRP3* or *STAT1:TNFRSF13B* reside in the same cellular biological pathways (Figure 2), suggesting a blend of 2 monogenic disease phenotypes.

Review of these variants reveals that *NLRP3* p.R490K, initially described as pathogenic causing a periodic fever syndrome,[39] is also reported to show reduced clinical penetrance in some individuals.[62] In P7, the clinical phenotype does contain traits that are supportive of an autoinflammatory syndrome with episodic fever and raised intracranial pressure (Table S4). This clinical information suggests that this variant is at least partially penetrant in this case. P7's clinical phenotype is likely to the result of a pathogenic *GATA2* variant with an additional *NLRP3* variant resulting in a blended phenotype of 2 distinct monogenic PIDs. Given conflicting literature reports of the pathogenicity of the *NLRP3* p.R490K variant, functional investigation is needed in this individual to clarify the role of this variant. Identification of *GATA2* haploinsufficiency merits consideration of bone marrow transplant; however, at the time of identifying this variant P7's health has deteriorated to condition that bone marrow transplantation was not suitable. Bone marrow transplantation is being increasingly used in adults with PID and knowledge of the monogenic cause for PID is useful in risk stratification for transplantation.

In P26, we consider that these variants may have also resulted in an overlapping blended phenotype as there is no direct interaction or common pathway between *STAT1* and *TNFRSF13B* (Figure 2). In P26, the variants *STAT1* p:V266I and *TNFRSF13B* p.Q57H have both been reported to be monogenic pathogenic with supportive functional data within the literature.[37,40] However, another report of *STAT1* p.V277I including a different assessment of phosphorylation studies of *STAT1* p.V266I did not show any hyperfunctional activity. These conflicting reports mean that one must question whether this is truly a pathogenic GOF variant, or a variant displaying incomplete penetrance in some individuals.[63] An additional report of *STAT1* p.V266I in a patient with disseminated mycobacteria is also not typical of the *STAT1* GOF phenotype (although mycobacterial disease is reported in *STAT1* GOF cohorts[47]), but would support the case that this variant impacts on STAT1 protein function in vivo.[64] Furthermore, the minor allele frequency of *STAT1* p.V266I (ExAC MAF = 0.002043) is above what may expect given the prevalence of *STAT1* GOF PID. Despite these factors, assessment of the variant against ACMG criteria arrives at the conclusion that it is pathogenic (Table 1), showing weight that published literature has on ACMG grading of genetic variants, as well as emphasising that one must apply a high level of scrutiny to literature and consider clinical phenotype when reviewing genetic variants.

TNFRSF13B variants present a unique challenge in patients with the PID; common variable immunodeficiency (CVID), such as P26. Originally reported to be associated with CVID,[65–67] *TNFRSF13B* variants have been reported as monogenic causes of CVID, with limited additional functional data to support them as a monogenic cause of PID.[68,69] Large-scale studies of the genomics of populations have found that *TNFRSF13B* variants are present at too high a MAF within healthy individuals to be considered monogenic causes of PID. *TNFRSF13B* variants do appear to be genetic risk factors for developing CVID and also convey an increased risk of autoimmunity in patients with CVID.[48] Due to this increased risk of autoimmunity in patients with CVID, knowledge of *TNFRSF13B* variants is important for clinical management of patients, having implications for disease monitoring. This is reflected in our case, where despite literature reports and the reported low ExAC MAF of the variant, the ACMG classification only grade *TNFRSF13B* p.Q57H as a VUS.

The phenotyping of PID patients often requires detailed, bespoke, functional immunological assays to determine specific defects in cellular functions.[70] These data are difficult to accurately reflect in HPO terms which are often better suited to describe physical characteristics. Often the lack of a specific HPO term for a laboratory assay result (eg, impairment of specific T cell subset such as Th17 cells) means that a broader term such as "impaired T cell function" (HP:0005435) or "abnormality of interleukin secretion" (HP:0011117) must be substituted. This use of broader HPO terms means that, via semantic relationships used by the *Phenomizer* algorithm, an excessive number of candidate genes are returned which may be missing the correct genetic diagnosis once a $P < .05$ to correct for random sampling is applied (Table S4). Due to these current limitations in HPO terminology, a broad unbiased approach of interrogating all known PID genes appears to result in a better diagnostic yield than reliance on HPO–term-driven candidate gene lists. There appears a need to add additional HPO terms and data to improve these results for PID diagnostics in the future.

In conclusion, this study shows the clinical application and benefits that genetic testing has in the clinical care of PID. PID is particularly amenable to precision medicine, and accurate diagnostics readily translate into effective treatments. We show that this 242 PID gene panel can provide clinically important information for management of PID in the clinic. However, it also highlights some of the limitations and disadvantages of gene panels in the rapidly moving field of PID genomics and the current challenges in accurate HPO term assignment for PID phenotyping.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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