# Utilization of next-generation sequencing to define the role of heterozygous *FOXN1* variants in immunodeficiency



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**Background: Forkhead box protein N1 (FOXN1) transcription factor plays an essential role in the development of thymic epithelial cells, required for T-cell differentiation, maturation, and function. Biallelic pathogenic variants in *FOXN1* cause severe combined immunodeficiency (SCID). More recently, heterozygous variants in *FOXN1*, identified by restricted gene panels, were also implicated with causing a less severe and variable immunodeficiency.**

**Objective: We undertook longitudinal follow-up and advanced genetic investigations, including whole exome sequencing and whole genome sequencing, of newborns with a heterozygous variant in *FOXN1*.**

**Methods: Five patients (3 female, 2 male) have been followed since they were first detected with low T-cell receptor excision circles during newborn screening for SCID. Patients underwent immune evaluation as well as genetic testing, including a primary immunodeficiency panel, whole exome sequencing, and whole genome sequencing in some cases.**

**Results: Median follow-up time was 6.5 years. Initial investigations revealed low $CD3^+$ T lymphocytes in all patients. One patient presented with extremely low lymphocyte counts and depressed phytohemagglutinin responses leading to a tentative diagnosis of SCID. Over a period of 2 years, $CD3^+$ T-cell counts rose, although in some patients it remained borderline low. One of 5 children continues to experience recurrent upper respiratory infections and asthma episodes. The remaining are asymptomatic except for eczema in 2 of 5 cases. Lymphocyte proliferation responses to phytohemagglutinin were initially low in 3 patients but normalized by age 10 months. In 3 of 5 cases, T lymphocyte counts remain low/borderline low.**

**Conclusion: In cases of monoallelic *FOXN1* variants, using whole exome sequencing and whole genome sequencing to rule out possible other significant pathogenic variants allowed us to proceed with confidence in a conservative manner, even in extreme cases consistent with newborn screen–positive early presentation of SCID. (J Allergy Clin Immunol Global 2024;3:100267.)**

***Key words:*** *Severe combined immunodeficiency, thymus, FOXN1, T-cell receptor excision circles, newborn screening, whole exome sequencing, whole genome sequencing*

The forkhead box protein N1 (FOXN1, or winged-helix-nude) transcription factor is an essential regulator of skin and thymic epithelial cell function.[1] The thymus provides a unique environment to support the maturation of progenitor T cells, from triple-negative $CD3^-CD4^-CD8^-$ thymocytes to single positive $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells bearing receptors for antigen recognition. Development of a diverse T-cell receptor repertoire, arising from V(D)J recombination, is dependent on the spatiostructural organization of the thymic stroma—including cortical thymic epithelial cells and medullary thymic epithelial cells required for positive and negative selection, respectively. T-cell–derived cues are likewise essential for proper thymic epithelial cell development, and aberrations in thymic architecture are observed in immunodeficiencies associated with stunted thymopoeisis.[2] Notably, the expression of FOXN1 and subsequent transcriptional regulation of downstream genes[3-6] are critical for the proper development and differentiation of cortical thymic epithelial cells and medullary thymic epithelial cells pre- and postnatally.[1,7,8] The absence or functional loss of FOXN1 results in athymia, ablated T-cell development, and the phenotype of severe combined immunodeficiency (SCID).

Null biallelic variants in the *FOXN1* gene, encoding FOXN1, are characterized by $T^-B^+NK^+$ SCID, alopecia universalis, and nail dystrophy (OMIM 601705).[9-11] Patients experience recurrent infections, failure to thrive, and oral candidiasis. To date, 5 distinct homozygous pathogenic variants in *FOXN1* leading to this phenotype have been described, including R255X,[10-13] R320W,[12,14] S188fs,[10,15] R114X,[14] and V294I.[16] Affected individuals require corrective hematopoietic stem cell transplantation (HSCT) or thymus transplantation to survive beyond the early years of

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*Abbreviations used*
FOXN1: Forkhead box protein N1
HSCT: Hematopoietic stem cell transplantation
NK: Natural killer
PHA: Phytohemagglutinin
PID: Primary immunodeficiency
SCID: Severe combined immunodeficiency
TCAG: The Centre for Applied Genomics
TCRVβ: T-cell receptor V beta
TREC: T-cell receptor excision circles
WES: Whole exome sequencing
WGS: Whole genome sequencing

life.[10,12,14] Biallelic compound heterozygous variants presenting with immunodeficiency and thymic aplasia have also been reported.[14,17] Recently, monoallelic variants in *FOXN1* (Fig 1) have been implicated in causing a variable clinical phenotype ranging from mild lymphopenia to possible profound T-cell immunodeficiency allegedly requiring HSCT.[6,14] Indeed, FOXN1 is characterized by partial genetic constraint to heterozygous truncating variants (gnomAD v2.1.1 o/e pLOF = 0.13, 90% confidence interval 0.06-0.35), which is consistent with a dominant haploinsufficient mechanism with reduced penetrance.

Newborn screening for SCID has transformed the care of patients with the most severe forms of primary immunodeficiency (PID), allowing early diagnosis and treatment before life-threatening infections and complications begin.[18] Screening utilizes the measurement of T-cell receptor excision circles (TRECs), which are a by-product of V(D)J recombination.[19,20] Because TREC levels directly reflect maturation of T cells in the thymus, they serve as a useful measure of T-cell development.[21] Notably, patients with SCID have very low/absent TREC levels,[20] and those with other forms of PID, including aberrations arising from *FOXN1,*[6] may also be detected by this method.[22-24]

Remarkably, most reported cases of PID due to heterozygous pathogenic changes in *FOXN1,* especially the more severe ones in patients who underwent HSCT, have not been evaluated for a putative second *FOXN1* variant or another genetic change associated with profound T-cell deficiency beyond a limited targeted PID gene panel. To explore these possibilities, we have expanded studies to whole exome sequencing (WES) and whole genome sequencing (WGS) in a cohort of patients with a monoallelic variant in *FOXN1*.

## METHODS

### Data collection

Patients included in this study were flagged for immune investigations after an abnormal newborn screen result for SCID. Guardian consent was obtained for enrollment in the Canadian Centre for Primary Immunodeficiency Registry and Tissue bank, approved by The Hospital for Sick Children's research ethics board (protocol 1000005598). Patient data, including demographics and laboratory evaluations, were collected from medical charts.

### Genetic analyses

Detailed methodology for genetic analyses can be found in this article's Online Repository available at www.jaci-global.org.

### Sample preparation and sequencing

For the genomes of patient (P) P1 and P5, about 1 μg of genomic DNA was submitted to The Centre for Applied Genomics (TCAG, The Hospital for Sick Children); 700 ng DNA was used as input material for library preparation using the Illumina TruSeq PCR-free DNA Library Prep Kit following the manufacturer's recommended protocol. Validated libraries were then pooled in equimolar quantities and paired-end sequenced on an Illumina HiSeq X platform following Illumina's recommended protocol to generate paired-end reads of 150 bases in length and about 100 gigabases of raw data per library. For exomes of P1, P2, and P3, good-quality genomic DNA samples were sent to TCAG for exome library preparation using Agilent SureSelect Human Exome Library Preparation V5 kit using the Agilent Bravo Automation System and paired-end sequencing on a HiSeq 2500 platform. For the exomes of P4 and P5, sequencing was performed at The Hospital for Sick Children's Department of Paediatric Laboratory Medicine using an Illumina NextSeq 500 instrument after enrichment with Agilent SureSelect Clinical Research Exome v1, with 2 × 150 bp reads.

### Alignment and variant calling

For the genomes of P1 and P5, reads were mapped to the b37 reference sequence using the BWA-MEM algorithm. Duplicate reads were marked using Picard Tools. Local realignment and base quality score recalibration were performed using GATK 3.7. Variants were called using HaplotypeCaller (GATK 3.7).[25] CNVs, comprising losses and gains with size ≥1 kb, were called using a pipeline[26] based on the read depth callers ERDS[27] and CNVnator.[28] Gains of size 1-5 kb supported only by ERDS were included. For exomes of P1, P2, and P3, reads were aligned to the hg19 build human reference genome using BWA-0.5.9.[29] PCR duplicates were marked using picard-tools-1.108, and local realignment and base recalibration were performed using GATK 1.1-28. Variants (single nucleotide variant/indel) were called using GATK UnifiedGenotyper 1.1-28.[30] For the exomes of P4 and P5, alignment was performed using BWA 0.7.15 and variant calling using GATK 3.6.0.

### Variant annotation and prioritization

We prioritized substitutions and small insertion/deletion (single nucleotide variant/indel) variants that were rare and that affected exonic sequence directly, or that could affect it indirectly by altering splicing or other regulatory sequences. We then sorted rare variants into 5 groups according to mode of inheritance (homozygous, X-chromosome hemizygous, potentially compound heterozygous, autosomal dominant, and potentially dominant haploinsufficient genes), applying more conservative frequency cutoffs for the dominant and X-linked groups; finally, we flagged genes implicated in PID (400 genes) or predicted to have potential implication in PID (2402 additional genes at a 80% recall cutoff). This prioritization approach was previously described.[31,32]

### Immune evaluation

**Lymphocyte proliferation.** Lymphocyte proliferative responses to the mitogen phytohemagglutinin (PHA) were determined by H-thymidine incorporation.[33,34] All assays were

performed in triplicate and compared to simultaneously stimulated cells from healthy control subjects.

**Serum concentration of immunoglobulins and specific antibodies.** Serum concentrations of immunoglobulins (IgG, IgA, and IgM) were measured using nephelometry and levels of serum tetanus toxoid were measured by means of ELISA, according to the manufacturer's instructions (Binding Site).

## RESULTS

### Patient characteristics and family history

Five infants (3 female, 2 male) who had positive newborn screening results for SCID were included in this study and followed at our center from a median age of 7 weeks (range, 2 weeks to 6 months) to 6.5 years (range, 2.5-8 years). All were born to nonconsanguineous parents. P1 (female) was born at 38 weeks after an unremarkable pregnancy and delivery. Her parents are of Italian descent with 2 healthy older children; her mother has a history of eczema, arthritis, and a thyroid goiter, with further maternal family members affected by Hashimoto thyroiditis, Hodgkin lymphoma, eczema, asthma, and celiac disease. Her father is healthy. P2 (male) was born at 40 + 5 weeks after an unremarkable pregnancy to parents of European descent. His mother had 3 previous miscarriages. The family history is significant for recurrent upper respiratory tract infections in the maternal grandmother and aunt, as well as type 1 diabetes mellitus in the maternal grandfather. The child's mother has eczema and father has chronic urticaria. P3 (female) was born at 39 + 2 weeks after an uncomplicated spontaneous vaginal delivery and an unremarkable pregnancy. Her parents are of mixed English, Dutch, and African American descent. Her father had a tonsillectomy and adenoidectomy as a child for recurrent tonsillitis, and he experienced a number of acute otitis media infections until the age of 4 years for which he did not require any surgical intervention. Her mother is healthy, as are 2 maternal half-siblings. P4 (male) was born at 37 weeks after cesarian section for placenta previa. The pregnancy was the product of *in vitro* fertilization and was otherwise only remarkable for maternal receipt of inhaled corticosteroids to treat asthma. His parents are of Ashkenazi Jewish descent, and family history is remarkable for asthma and eczema in both his mother and older brother. P5 (female) was followed by our center since birth. She is the first child born to parents of Asian descent. The pregnancy was uncomplicated, and she was born at 39 + 2 weeks by spontaneous vaginal delivery. Her mother had 2 prior early miscarriages, and her father has 2 healthy older children from a previous marriage.

### Newborn screening for SCID

During newborn screening for SCID, dried blood spot samples collected at day 1 of life are evaluated for TREC levels, an indication of T-cell maturation.[19] Infants with TREC levels below the cutoff of 75 copies/3 μL are flagged positive for SCID and undergo a repeat TREC determination 7 days after the first test. Of the 5 infants in our cohort, only 1 (P5) had an initial TREC level of zero (repeat: 7 copies/3 μL). Two remained low/borderline low at repeat measurement (P1—initial: 28.8 copies/3 μL, repeat: 61.8 copies/3 μL; P3—initial: 48 copies/3 μL, repeat: 41.3 copies/3 μL), while 2 later recorded normal TREC levels (P2—initial: 60.6 copies/3 μL, repeat: whole blood TREC analysis—1135 copies/0.50 μg [normal]; P4—initial: 40 copies/3 μL, repeat: 138 copies/3 μL).

### Clinical manifestations

Of the 5 infants, only P1 experienced recurrent infections, including 2 upper respiratory tract infections and 1 bout of pneumonia requiring oral antibiotics occurring before the age of 6 months. She subsequently had an influenza A infection requiring admission to hospital for oseltamivir treatment. She was also diagnosed with asthma requiring inhaled as well as periodic systemic steroid therapy. Now aged 8 years, she continues to endure 3 or 4 upper respiratory infections per year as well as periodic asthma exacerbations. She is otherwise growing well and has never had any deep-seated, fungal, viral, or opportunistic infections. P2, P3, P4, and P5 were clinically well at the time of presentation. P2 (aged 7.5 years) and P4 (aged 5 years), who both have mild eczema, are well and thriving, with no infectious, inflammatory, or autoimmune manifestations. P3 (aged 6.5 years) continues to remain well with no apparent signs or symptoms. No patients or their parents exhibited alopecia or nail dystrophy.

### Immune evaluation

All 5 patients had $CD3^+$ T-cell lymphopenia at presentation, including $CD3^+CD4^+$ cells (Table I). Four of 5 (P1, P3, P4, P5) also had low $CD3^+CD8^+$ cells. Notably, T-cell counts were profoundly low in P5 with $CD3^+CD4^+$ cells at 43 cells/μL and $CD3^+CD8^+$ cells at 5 cells/μL. $CD16^+/CD56^+$ natural killer (NK) cells were initially elevated in 1 of 5 cases (P4). Lymphocyte proliferative responses to the mitogen PHA were low in 2 infants (P1, P2) and profoundly low in P5. T-cell receptor V beta (TCRVβ) analysis (Fig 2) revealed abnormal representation of a subset of Vβ clones in P1, P2, and P3 (results are unavailable for P4). A mild overrepresentation of Vβ 1 in $CD3^+CD8^+$ cells of P1 and P2 was noted. In P3, Vβ 2 was overrepresented in $CD3^+CD4^+$ cells, whereas Vβ 7.1 was overrepresented and Vβ 18 absent from $CD3^+CD8^+$ cells. Notably, skewed TCRVβ repertoire was reported in P5, with overrepresentation of Vβ 3, 5.1, 12, and 23, underrepresentation of Vβ 13.2 and 21.3, and complete absence of Vβ 7.2 in $CD3^+CD4^+$ cells. In $CD3^+CD8^+$ cells, marked overrepresentation of Vβ 3, 7.1, and 13.1, and absence of both Vβ 7.2 and 18 was found.

Overall, the extremely low T-cell counts, poor T-cell function, and skewed TCRVβ repertoire in P5 led to a diagnosis $T^-B^+NK^+$ SCID, triggering additional workup for HSCT. Output of naive T cells in this patient was low at 9% ($CD4^+$/$CD45RA^+$), while memory T cells predominated at 84% ($CD4^+$/$CD45RO^+$). Recent thymic emigrants were also low (9%; reference range, 64-94%). Chest imaging revealed an absent thymic shadow. She received preventative therapy at 1 month of age, including immunoglobulin replacement therapy and prophylaxis for *Pneumocystis jirovecii* pneumonia. In P1, P2, and P3, output of naive T cells was similar to laboratory healthy controls (measured within the first year of life). $CD4^+/CD45RA^+$ and $CD4^+/CD45RO^+$ testing was not performed in P4.

$CD19^+$ B-cell counts were slightly reduced in P1 while immunoglobulins and specific antibody titers were within reference range. P2, P3, P4, and P5 exhibited normal B-cell counts and age-appropriate immunoglobulin antibody titers.

**TABLE I.** Longitudinal immune evaluation of heterozygous *FOXN1* cases

| Characteristic | P1 | | P2 | | P3 | | P4 | | P5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| TREC | | | | | | | | | | |
| First | 28.8 copies/3 μL | | 60.6 copies/3 μL | | 48 copies/3 μL | | 40 copies/3 μL | | 0 copies/3 μL | |
| Second | 61.8 copies/3 μL | | 1135 copies/0.5 μg | | 41.3 copies/3 μL | | 138 copies/3 μL | | 7 copies/3 μL | |
| Phenotype | Recurrent respiratory infections, asthma | | Eczema | | Asymptomatic | | Eczema | | Asymptomatic | |
| **Evaluation age** | **First (6 months)** | **Last (80 months)** | **First (10 weeks)** | **Last (12 months)** | **First (7 weeks)** | **Last (56 months)** | **First (3 weeks)** | **Last (33 months)** | **First (2 weeks)** | **Last (25 months)** |
| Leukocytes ($\times 10^9$/L) | 3.4 (L) [5.0-15.0] | 6.00 [4.23-9.99] | 6.9 [5.0-20.0] | 7.1 [5.0-15.0] | 6.6 [5.0-20.0] | 8.66 [4.86-13.8] | 8.86 [7.8-15.91] | 4.94 (L) [5.75-13.50] | 5.35 (L) [8.16-14.56] | 4.65 (L) [5.75-1350] |
| Hemoglobin (g/L) | 109 [100-140] | 133 [12-141] | 118 [90-135] | 122 [100-140] | 119 [115-180] | 118 [102-127] | 97 (L) [100-153] | 123 [100-132] | 160 [134-200] | 98 (L) [100-132] |
| Platelets ($\times 10^9$/L) | 288 [150-400] | 265 [203-431] | 524 (H) [150-400] | 335 [150-400] | 463 (H) [150-400] | 398 [189-384] | 306 [248-586] | 233 [203-431] | 344 [144-449] | 339 [203-431] |
| Lymphocytes ($\times 10^9$/L) | 1.87 (L) [4.0-10.5] | 2.05 [1.34-4.12] | 4.69 [2.0-17.0] | 4.89 [4.0-10.5] | 2.55 [2.0-17.0] | 2.68 [1.25-5.77] | 5.14 [2.11-8.38] | 2.25 [1.9-6.30] | 0.75 (L) [1.75-8.0] | 1.31 (L) [1.90-6.30] |
| Monocytes ($\times 10^9$/L) | 0.17 [0.05-1.10] | 0.51 [0.27-0.81] | 0.55 [0.20-2.40] | 0.68 [0.05-1.10] | 0.92 [0.20-2.40] | 0.64 [0.24-0.92] | 1.42 (H) [0.28-1.38] | 0.43 [0.37-1.45] | 1.59 [0.57-1.72] | 0.63 [0.37-1.45] |
| Neutrophils ($\times 10^9$/L) | 0.95 (L) [1.5-8.5] | 3.29 [1.45-6.75] | 1.24 [1.0-9.5] | 1.34 [1.5-8.5] | 2.88 [1.0-9.50] | 5.15 [1.6-8.29] | 2.04 [1.18-5.45] | 2.13 [1.45-6.75] | 2.58 [1.73-6.75] | 2.54 [1.45-6.75] |
| Basophils ($\times 10^9$/L) | ND | 0.03 [0.01-0.06] | 0.07 [0.0-0.20] | 0.0 [0.0-0.2] | 0.06 [0.0-0.20] | 0.02 [0.0-0.20] | 0.0 (L) [0.01-0.07] | 0.02 [0.01-0.06] | 0.0 (L) [0.02-0.07] | 0.01 [0.01-0.06] |
| Eosinophils ($\times 10^9$/L) | 0.10 [0.05-0.70] | 0.11 [0.06-0.97] | 0.35 [0.07-1.0] | 0.14 [0.05-0.7] | 0.17 [0.07-1.0] | 0.13 [0.03-0.46] | 0.18 [0.03-0.61] | 0.10 [0.0-0.79] | 0.1 [0.09-0.64] | 0.15 [0.0-0.79] |
| $CD3^+$ total (cells/μL) | 1053 (L) [1600-6700] | 1338 [1239-2611] | 1670 (L) [2300-6500] | 2254 [1600-6700] | 1040 (L) [2300-6500] | 1274 (L) [1578-3707] | 2270 (L) [3180-5401] | 1426 (L) [1578-3707] | 46 (L) [3180-5401] | 483 (L) [1578-3707)] |
| $CD3^+CD4^+$ total (cells/μL) | 802 (L) [1400-5100] | 713 [646-1515] | 1037 (L) [1500-5000] | 1352 [100-4600] | 837 (L) [1700-5300] | 850 (L) [870-2144] | 1583 (L) [2330-3617] | 797 (L) [870-2144] | 43 (L) [2330-3617] | 373 (L) [870-2144] |
| $CD3^+CD8^+$ total (cells/μL) | 214 (L) [600-2200] | 392 [365-945] | 587 [500-1600] | 813 [400-2100] | 178 (L) [400-1700] | 284 (L) [472-1107] | 667 (L) [712-1361] | 546 [472-1107] | 5 (L) [712-1361] | 91 (L) [472-1107] |
| $CD19^+$ total (cells/μL) | 638 (L) [776-2238] | 258 (L) [276-640] | 1825 [600-3000] | 1166 [600-2700] | 903 [600-1900] | 1122 [434-1274] | 1380 (315-1383) | 468 [434-1274] | 431 [315-1383] | 656 [434-1274] |
| $CD16^+CD56^+$ total (cells/μL) | 358 [100-1000] | 511 (H) [120-483] | 981 [100-1300] | 1303 [200-1200] | 438 [200-1400] | 408 [155-565] | 1815 (H) [201-870] | 304 [155-565] | 622 [201-870] | 318 [155-565] |
| $CD4^+/CD45RA^+$ (%) | 47.8 | ND | ND | 33.6 | 22.6 | ND | ND | ND | 9.0 | 31.1 |
| $CD4^+/CD45RO^+$ (%) | 12 | | | 12.6 | 11.1 | | | | 84.0 | 65.3 |
| IgG (g/L) | 2.8 [1.1-7.0] | 5.8 [5.4-13.6] | 2.2 [1.1-7.0] | 2.8 [1.1-7.0] | ND | 7.8 [3.2-11.5] | ND | 3.9 [3.2-11.5] | 6.0 [1.1-7.0] | 5.5 [3.2-11.5] |
| IgA (g/L) | 0.1 [0.0-3.0] | 0.4 (L) [0.5-2.2] | 0.1 [0.0-3.0] | 0.1 [0.0-0.3] | 0.1 [0.0-0.3] | 0.4 [0.0-0.9] | ND | 0.4 [0.0-0.9] | 0.1 [0.0-0.3] | 0.3 [0.0-0.9] |

*(Continued)*

**TABLE I.** *(Continued)*

| Evaluation age | First (6 months) | Last (80 months) | First (10 weeks) | Last (12 months) | First (7 weeks) | Last (56 months) | First (3 weeks) | Last (33 months) | First (2 weeks) | Last (25 months) |
|---|---|---|---|---|---|---|---|---|---|---|
| IgM (g/L) | 0.7 [0.2-0.9] | 0.7 [0.5-1.9] | 0.4 [0.1-0.7] | 0.6 [0.2-0.9] | 0.3 [0.1-0.7] | 1.2 [0.5-1.9] | ND | 0.3 [0.4-1.5] | 0.5 [0.1-0.7] | 0.9 [0.5-1.9] |
| Anti-diphtheria toxoid IgG (IU/mL) | ND | 0.86 | ND | NA | ND | 1.22 | ND | 0.39 | ND | 0.89 |
| Anti-tetanus toxoid IgG (IU/mL) | 1.69 | 2.00 | ND | 4.15 | ND | 2.71 | ND | 2.96 | ND | 4.30 |
| T-cell PHA stimulation index [normal > 400] | 98.2 (L) | 3715 | 187.2 (L) | 466.1 | 906 | 1429 | ND | 1594 | 12 (L) | 481.7 |

Reference ranges appear in brackets. *NA*, Not applicable; *ND*, not done.

Our patients were followed for a median period of 6.5 years (range, 2.5-8 years). At present, 3 of 5 children remain lymphopenic with low or borderline low total $CD3^+$ cells (including $CD3^+CD8^+$ and/or $CD3^+CD4^+$ T cells; Fig 3, *A* and *B,* Table I) in P3, P4, and P5. In P1, $CD3^+CD4^+$ and $CD3^+CD8^+$ counts continue to fluctuate, although they are currently within reference range. Her B-cell counts were initially borderline low and to date remain slightly reduced alongside borderline low IgA titers, but with normal IgG and IgM as well as normal antibody vaccine responses. P2's immune parameters normalized completely. In P4, $CD3^+CD8^+$ cell counts normalized from 4 months of age while $CD3^+CD4^+$ cell counts increased at around 1 year of age, although they also fluctuate. In the 3 of 5 children who had low initial lymphocyte proliferation to PHA, *in vitro* responses normalized beginning at 10 months of age (Fig 3, *C*). P1, P2, P3, and P4 have received all age-appropriate vaccines, with good antibody response and no complications associated with live/attenuated immunizations.

Notably, P5 (currently 2.5 years of age), in whom we deferred workup for HSCT, has shown improvement in her immune parameters, although they remain borderline low. Her CD45RA/RO assessment demonstrated improving naive T cells (31.10%) versus memory T cells (65.30%), and recent thymic emigrants continue to increase (21%). TCRVβ analysis shows improvement, although $CD3^+CD4^+$ cells still demonstrate mild overrepresentation of TCRVβ clone 3. Her $CD3^+CD8^+$ cells show overrepresentation of Vβ 3 and 14, and absence of Vβ 7.2 (Fig 2, *C*). Her immunoglobulin levels are currently within reference range. She is clinically well and has received her routine vaccines with the exception of live/attenuated vaccines.

## Genetics

Immunodeficiencies associated with *ADA, PNP, ZAP70,* and *IKBKB* were ruled out during initial routine newborn screening tests, and assessment for microdeletions in 22q11.6 by microarray was negative. Subsequently, next-generation sequencing encompassing targeted PID gene panels, WES, and WGS were utilized to identify the underlying genetic aberration in each case. We identified 2 missense, 1 splice-site, and 2 frameshift variants in *FOXN1* (NM_003593.2) (Table II, Fig 1). In P1, WES and WGS identified a novel heterozygous missense variant in exon 6 (of 8 total) of *FOXN1,* c.848C>T (p.A283V), resulting in substitution of an alanine residue with valine at position 283 of the forkhead domain. This variant was not reported in large population databases, including gnomAD, dbSNP, ClinVar, or HgMD, and is predicted *in silico* to be damaging (SIFT = 0, PolyPhen2 HVAR = 0.999, Mutation Assessor = 2.9). Sanger sequencing of the parents revealed that her father carried the same variant. In P2, a 20-gene SCID panel (Cincinnati Children's Hospital) and follow-up WES were used to identify a heterozygous missense mutation in exon 6 of *FOXN1,* c.958C>T (p.R320W), resulting in substitution of arginine with tryptophan at position 320 within the forkhead domain.[12] Segregation analysis confirmed the variant in both the patient and his father. In P3, WES revealed a novel heterozygous splice-site alteration predicted to cause frameshift exon skipping (c.927+1G>C). Her healthy and asymptomatic mother was found to harbor the same variant. In P4, a targeted PID gene panel (Blueprint Genetics) followed by WES revealed a single novel heterozygous frameshift-inducing deletion in the forkhead domain of *FOXN1,* c.1046del (p.N349Ifs*201). In P5, we utilized both

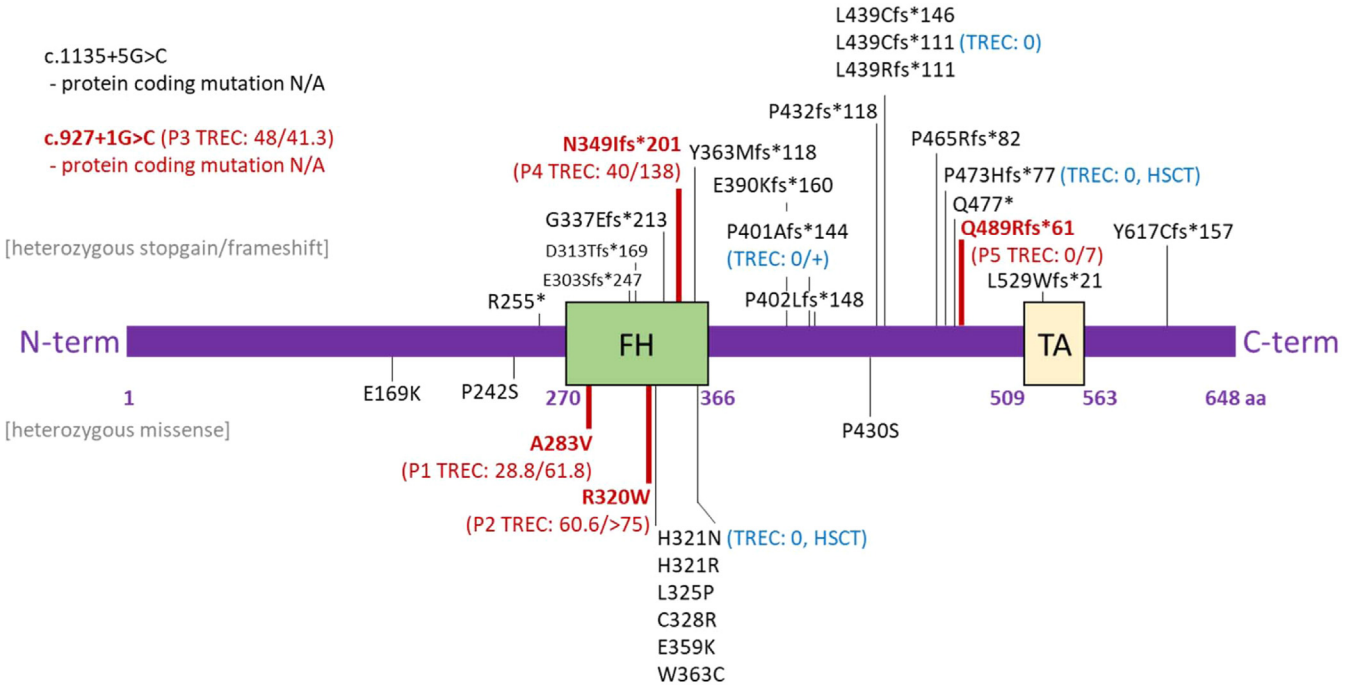


**FIG 1.** Schematic diagram of FOXN1 with location of heterozygous *FOXN1* variants. Transcription factor FOXN1 is composed of 648 amino acid residues encompassing $NH_2$-terminal region (N-term), DNA-binding forkhead (FH) domain, and COOH-terminal (C-term) region containing a transactivation (TA) domain. *FOXN1* variants and corresponding TREC levels identified after SCID newborn screening in our study are marked in *red*. Heterozygous stop-gain/frameshift variants appear in *upper* portion of schematic, with heterozygous missense variants in *lower* portion. Previously reported variants are shown in *black*. Variants associated with absent TREC levels (in previously reported studies) are shown in *blue*.

WES and trio WGS to identify a rare heterozygous frameshift mutation targeting exon 7 of *FOXN1*, c.1465del (p.Q489Rfs*61), arising from the substitution of glutamine with arginine at position 489, as the sole pathogenic variant.[17] This variant was absent from large population databases (gnomAD) and arose *de novo,* given that it was not present in either parent.

In each of these cases, the identification of a single heterozygous *FOXN1* variant and exclusion of other variants (see the Online Repository at www.jaci-global.org) allowed us to switch to a more conservative management plan.

## DISCUSSION

Newborn screening for SCID has revolutionized the ability to identify and treat infants with profound immune defects before infectious complications occur.[35] While the majority of flagged cases are the result of perinatal stress or maternal factors,[23] those that are confirmed with *bona fide* SCID require lifesaving intervention.[22] Early detection leads to expedited preparation for HSCT in cases of primary defects targeting lymphocyte growth and development, or more rarely in cases of nonhematopoietic thymic tissue defects. This urgency to proceed with a curative yet high-risk procedure should not rely solely on clinical or immune evaluations in the modern era.

A definitive molecular diagnosis is essential for prognosis, treatment, and counseling purposes. While targeted gene panels can detect variants within a predefined set of genes, these preclude the identification of defects in genes not yet captured in panels. Further, commercial panels typically pick up numerous monoallelic variants but may fail to reveal additional variants that are critical for the diagnosis of recessively inherited conditions. Expansive next-generation sequencing analysis, including WES and WGS, is advantageous and was shown to be of great value in the discovery of a number of PID-causing genes,[31,36,37] and it could assist in ruling out some recessive conditions.

As an excellent example, P5 presented with laboratory values indicative of SCID that could be caused by a biallelic *FOXN1* variant requiring stem cell or thymic transplantation. Yet only a heterozygous change was detected by the gene panel, raising the hypothesis that a second variant could have been missed. WGS excluded this possibility and swayed the medical decision from an urgent stem cell transplantation to a more conservative approach.[6,17,38] Indeed, both numbers and function of T cells improved over time, as expected of heterozygous *FOXN1* haploinsufficiency.

FOXN1, a member of the winged-helix domain transcription factors, is a 648–amino acid protein that regulates the expression of close to 500 genes.[8] Structurally, it is composed of an $NH_2$-terminal region, a DNA-binding forkhead domain, and a COOH-terminal region containing an evolutionarily conserved transcriptional activation domain (Fig 1).[39,40] Of the previously reported null variants, R255X results in loss of both the forkhead and transactivation domains, leading to the classic phenotype of $T^-B^+NK^+$ SCID with athymia, alopecia, and nail dystrophy.[10] Aberrations within the $NH_2$-terminal region (R114X,[14] S188fs[15]) or the forkhead domain (V294I,[16] R320W[12,14]) have been associated with features of SCID with or without nail

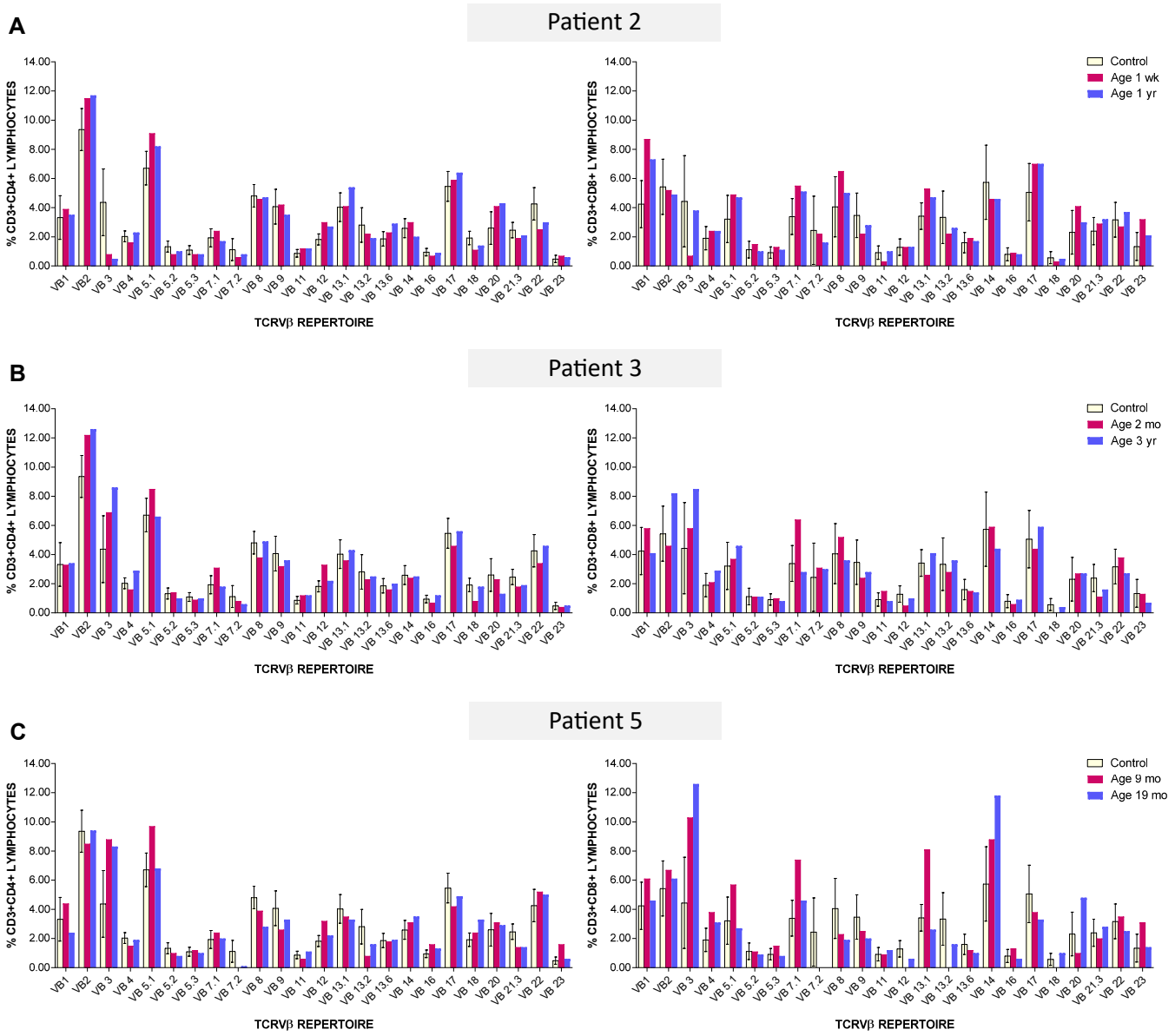


**FIG 2.** TCRVβ repertoire of patients with heterozygous *FOXN1* mutations. Flow cytometry–based assessment of TCRVβ clones was performed using lymphocytes of **(A)** P2, **(B)** P3, and **(C)** P5. Analysis of $CD3^+CD4^+$ cells and $CD3^+CD8^+$ cells are shown at *left* and *right,* respectively. *Open bars* indicate control reference rage; *magenta,* initial evaluation; and *blue,* last evaluation.

dystrophy. More recently, a 5–amino acid segment within the forkhead domain was shown to be essential for development of thymic epithelial cell without affecting the skin and nails.[17] Affected patients presented atypically with thymic aplasia and $T^-B^+NK^+$ SCID in the absence of keratinocyte abnormalities.

Heterozygous variants in *FOXN1* (Fig 1) have been shown to be associated with more variable clinical presentations. A case series of 25 children (including 21 newborns) harboring 19 distinct single *FOXN1* variants reported features of T-cell lymphopenia, including low $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells that partially recovered with age.[6] In contrast, a more severe clinical phenotype was reported in 2 siblings carrying the same heterozygous P465Rfs*82 mutation, reminiscent of the clinical picture of patients with biallelic *FOXN1* variants, in which one required HSCT.[14] However, WES/WGS were not used to rule out a potential second variant that could better explain the severe presentation. Further, it is plausible to assume that with time, the immune system would have spontaneously recovered, as was the case with our P5. In our study, P1 experienced recurrent infections and asthma, P2 and P4 had eczema, and P3 and P5 remained asymptomatic despite mild to severe lymphopenia and T-cell dysfunction. Notably, infants with an abnormal SCID newborn screen result are subject to greater protective measures, thus minimizing exposure to infections until a firm diagnosis is made.

The mutations identified in P1, P2, and P4 each target the evolutionarily conserved DNA-binding forkhead domain. The novel missense c.C848T (A283V) mutation in P1 introduces a moderate amino acid change and is predicted *in silico* to be deleterious. In P2, the missense c.C958T (R320W) variant results in

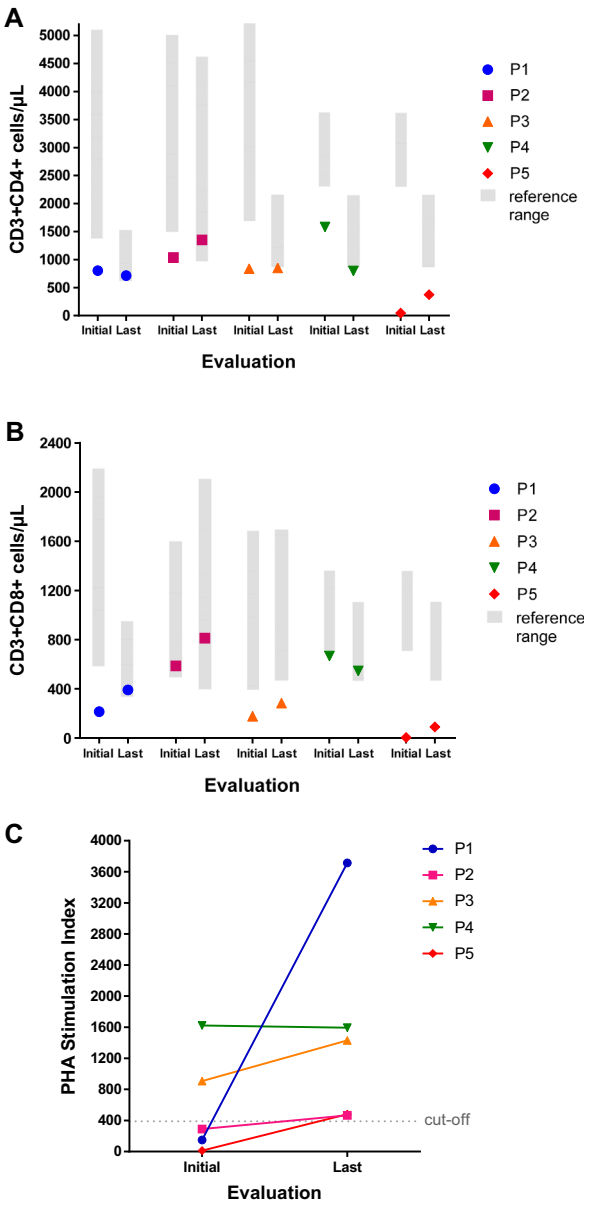


**FIG 3.** Longitudinal follow-up of immune parameters. **(A)** $CD3^+CD4^+$ and **(B)** $CD3^+CD8^+$ T-cell counts in cases P1 to P5 were followed over up to 80 months. Values at initial and last evaluation are shown. *Gray bars* indicate reference ranges. **(C)** Lymphocyte proliferation responses (stimulation index) to mitogen PHA during initial evaluation and last evaluation. Normal values are those with stimulation index (SI) >400 (cutoff, *dotted line*).

the replacement of a long-chain, positively charged amino acid (arginine) with a bulky aromatic residue (tryptophan) and was likewise predicted to be damaging. R320 binds the FOXN1 consensus sequence, and a biallelic form of the R320W variant was previously reported in a patient with SCID.[12] The c.1046del (N349Ifs*201) variant identified in P4 deletes 1 base pair to generate a frameshift, leading to a premature stop codon at position 201 (exon 8) in a new reading frame. This change is predicted to lead to the loss of 100 amino acids from the COOH terminal. Other variants resulting in the same size of truncated protein (548 amino acids) have been reported in multiple patients with T-cell lymphopenia.[6] In contrast, in P3, the novel c.927+1G>C variant eliminates a donor splice site that is perfectly conserved in vertebrates, which may result in exon skipping causing a frameshift, the possibility of alternative splice-site usage with exon truncation or extension causing a frameshift/stop-gain, or alteration of the forkhead protein domain. Only 1 other splice-site mutation in *FOXN1* has previously been reported; the female infant presented with T- and B-cell lymphopenia, gastroenteritis, and thymic aplasia.[6]

The frameshift mutation identified in P5, c.1465del (Q489Rfs*61), causes a premature stop within the COOH-terminal domain. This is predicted to result in loss of normal protein function due to truncation of the protein or nonsense-mediated mRNA decay, and was previously reported in 3 patients.[17] Two were in a monoallelic state and 1 in a compound heterozygous state, with a P340S polymorphism affecting the second allele. The patient harboring the Q489Rfs*61/ P340S variants presented similarly to ours, with $T^-B^+NK^+$ SCID, but later underwent HSCT at 6 months of age. Luciferase reporter studies revealed the Q489Rfs*61 variant possessed only 18% residual transcriptional activity, whereas the P340S polymorphism retained 100% transcriptional activity. Longitudinal follow-up of P5 in our study revealed improvement of immune parameters, indicating that despite substantially depressed FOXN1 activity, there were still sufficient levels to allow recovery of T-cell numbers and function. In line with this, a gene dosage effect for FOXN1 has been proposed,[6] as has been observed for other genes associated with immunodeficiency.[41] Of note, review of the available literature reveals a striking level of clustering of missense variants within the DNA-binding forkhead domain, while stop-gain/frameshift variants appear distributed across the protein sequence (Fig 1).

Segregation analysis of our cases revealed, in line with the current literature, great variability in the phenotype of individuals bearing heterozygous *FOXN1* variants. In 2 of 5 cases, Sanger sequencing identified the same heterozygous *FOXN1* variant in a healthy, asymptomatic parent (P1, P3) with no history of immune abnormalities. Similar patterns were previously reported in other immunodeficiency conditions such as autoimmune lymphoproliferative syndrome caused by fatty acid synthase variants.[42,43] The missense variant found in P2 segregated to the patient's father, who reports chronic urticaria, a manifestation not previously noted in heterozygous carriers and thus unlikely related to this genetic finding. It is not understood why in some immunodeficiency disorders parents who carry the same pathogenic allele as their offspring would be asymptomatic, although other gene modifiers or environmental factors may play a role. Given the remarkable time-dependent improvement of immunity in heterozygous *FOXN1* cases, it would be plausible to speculate that in many, a complete recovery occurs despite partial haploinsufficiency of *FOXN1*. Still, asymptomatic carriers of *FOXN1* variants should be followed for longer periods of time and studied more carefully in the future. In contrast, the frameshift mutation identified in P5 was absent from both parents, indicating *de novo* emergence. Segregation analysis of P4 was not completed because of lack of availability of parental samples.

Notably, reported TREC levels (from below threshold to absent) also vary among those with heterozygous variants affecting the same domain, or even the same amino acid residue, with no apparent genotype correlation. In the present study, only P5 had an initial TREC level of zero, while levels in the other 4 patients (of whom 3 also harbored mutations within the

**TABLE II.** Genetic investigations and variants identified

| Patient | *FOXN1* variant | Description | Sequencing | Inheritance |
|---|---|---|---|---|
| P1 | c.848C>T (p.A283V) | Missense | WES, WGS | Father |
| P2 | c.958C>T (p.R320W) | Missense | WES, PID panel | Father |
| P3 | c.927+1G>C | Splice site | WES | Mother |
| P4 | c.1046del (p.N349Ifs*201) | Frameshift | WES, PID panel | Not available |
| P5 | c.1465del (p.Q489Rfs*61) | Frameshift | WES, trio WGS | *De novo* |

evolutionarily conserved forkhead domain) varied from 28.8 to 60.6 copies/3 μL. Bosticardo et al reported 5 infants with the identical P401Afs*144 variant.[6] One had TREC levels of zero accompanied by profoundly low CD3 (<100 cells/μL) and absent CD8 cells. Four patients had TREC levels recorded as "below state threshold," with CD3 cells ranging from 327 to 928 cells/μL and B cells of between 81 and 135 cells/μL. An additional adult bearing the same mutation reported no clinical manifestations. Because the variants in *FOXN1* were identified by targeted PID panel sequencing, it remains to be determined whether an additional mutation may contribute to the absent TREC levels. Indeed, Du et al describe 2 heterozygous *FOXN1* patients with further mutations in the *DOCK8* and *CHD7* genes.[17] Interestingly, WES analysis of 2 other cases at our center similarly identified additional mutations in *CHD7* and *WAS,* respectively, alongside *FOXN1* (unpublished observations). The clinical course of the patient with a novel homozygous mutation in the *WAS* gene and heterozygous stop-gain mutation in *FOXN1* was marked by severe clinical course, including thrombocytopenia, low CD8 cells, and poor T-cell responses to PHA, while the patient with the variant in *CHD7* experienced persistent lymphopenia. In each of these cases, the clinical phenotype and subsequent underlying cause of PID could be explained by the other variant.

In summary, this report highlights the importance of robust genetic investigations to delineate the underlying cause of immunodeficiencies detected by SCID newborn screening. Given the wide availability and falling cost of next-generation sequencing techniques, analysis using targeted gene panels should be considered just a first step toward attaining a definitive genetic diagnosis.

## DISCLOSURE STATEMENT

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