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## Letter to the Editor

## Improving the diagnostic efficiency of primary immunodeficiencies with targeted next-generation sequencing

### To the Editor:

With more than 430 genetic defects, primary immunodeficiencies (PIDs) or inborn errors of immunity are a heterogeneous group of rare genetic disorders affecting the immune system.<sup>1</sup> Unraveling the genetic etiology of the disease is crucial as it can guide the choice of the most appropriate treatment.<sup>2</sup> Moreover, following molecular characterization, families of patients can benefit from appropriate genetic counseling for screening of relatives and prenatal diagnosis, both of which contribute to early life-saving treatment and disease prevention. The extreme diversity of clinical phenotypes and genetic defects make diagnosis challenging, even for experienced clinicians and medical biologists. In recent years, next-generation sequencing (NGS) has largely replaced Sanger sequencing in clinical laboratories, particularly for the diagnosis of highly heterogeneous diseases, such as PIDs.<sup>3</sup> We chose targeted NGS over whole exome sequencing (WES) because it was still cheaper and has the advantage to focus on a customized set of relevant genes, avoiding many ethical issues and ensuring great overall coverage, a prerequisite in a diagnostic setting. Many studies have proven the efficacy of NGS as a first-line diagnostic tool in PIDs, but a few have undertaken a systematic copy number variation (CNV) analysis.<sup>4</sup> The aim of this study was to build and to validate a large customized NGS panel with a high and uniform coverage for an integrative CNV analysis that could be used as a first-line tool for the diagnosis of PIDs.

We used a customized SureSelectXT Target Enrichment System (Agilent, Santa Clara, Calif) and the Illumina HiSeq 2500 Sequencing System (Illumina, San Diego, Calif). The bait territory was large, with targeted regions encompassing 300 genes and 1095 Mbp (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The mean depth of coverage reached  $539\times \pm 203\times$  per base and 99% of the targeted bases had coverage of more than  $30\times$ . To estimate the sensitivity of the method, we included 33 individuals (patients with PIDs and their relatives) with known disease-causing mutations, and we referred to them as positive controls. Two other relatives who do not carry the familial disease-causing mutation were included as negative controls. We were able to detect 38 of the 39 expected mutations (97%), including 1 large heterozygous CNV in *DOCK8* ( $\Delta 3-48$ ) and 8 small deletions ranging from 1 to 21 bp (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Due to the presence of pseudogene, additional analysis was required to detect 1 hemizygous mutation in *IKBKG*.

Between 2015 and 2018, we recruited prospectively 129 patients with a suspected inherited PIDs of unknown genetic etiology (Table I). We included patients for which there were numerous candidate genes or a candidate gene difficult to explore with conventional methods (see Table E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).<sup>1</sup> This targeted NGS strategy identified 46 different variants worth being reported in 40 of the 129 patients (31%) with no prior genetic diagnosis (Fig 1, A and B; see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Half of these variants have never been reported

TABLE I. Characteristic of PIDs cohort included in this study

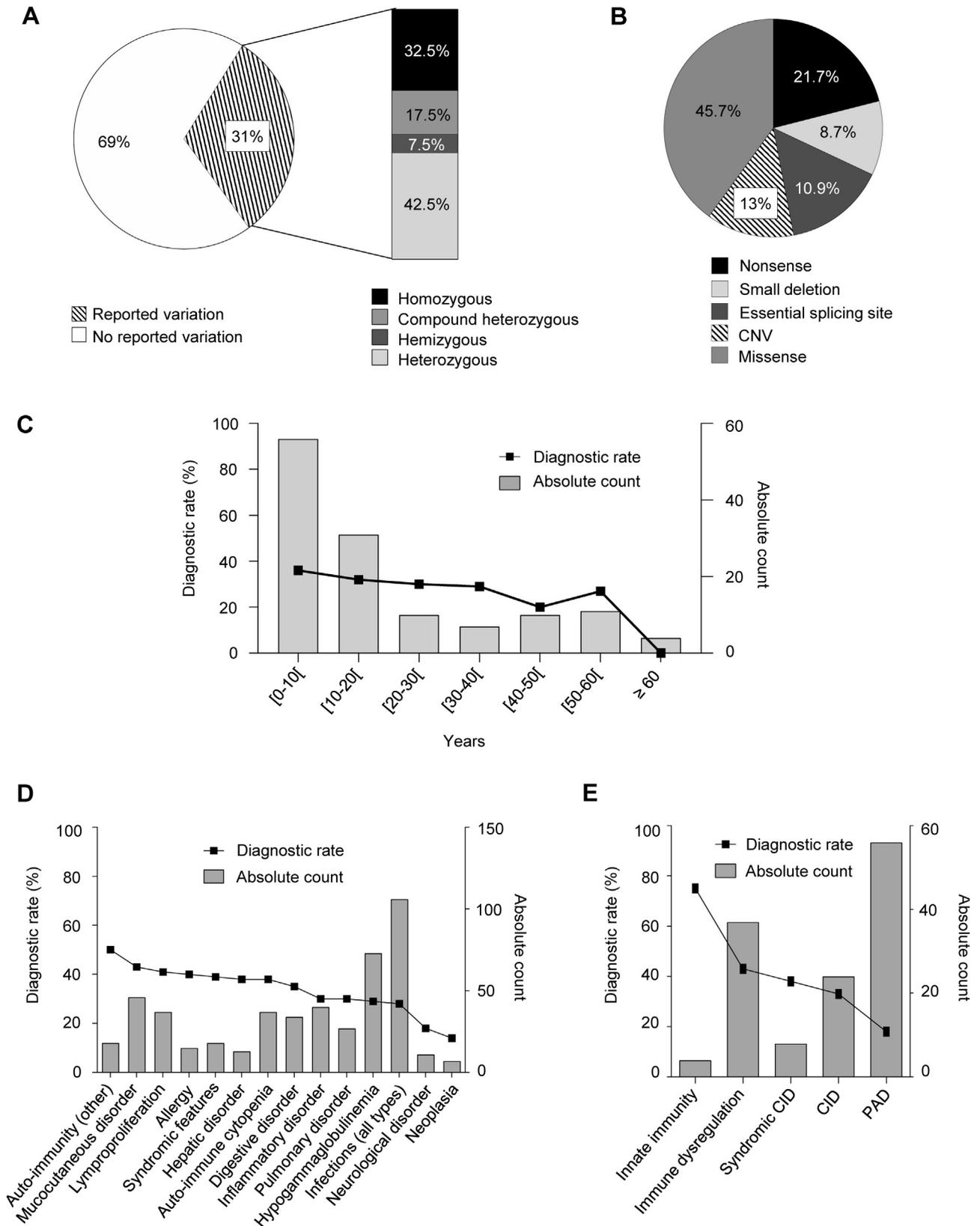
Characteristic	PIDs cohort
No. of patients	129
Age (y), median (range)	12.8 (0-72)
Pediatric patients (<18 years old)	64 (83)
Consanguinity	16 (21)
Sex ratio F/M	1:1
Previous unsuccessful genetic explorations	47 (61)
IUIS classification	
CID	19 (24)
CID with syndromic features	6 (8)
Primary antibody deficiency	43 (56)
Disease of immune dysregulation	29 (37)
Innate immune deficiency	3 (4)

CID, Combined immunodeficiency; F, female; IUIS, International Union of Immunological Societies; M, male.

Values are percentage (n) unless otherwise indicated.

in other patients or in the public databases. According to the American College of Medical Genetics criteria,<sup>5</sup> 41 were classified as pathogenic, 3 were likely pathogenic, and 2 were variants of unknown significance but could account for manifestations observed in the patients (see Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Diagnostic rate decreased with age but remained above 20% in all but the oldest group (Fig 1, C). This suggests that adult patients with diagnostic of PIDs should be genetically investigated especially when disease onset occurred in childhood. We then investigated the proportion of patients for which we retained a diagnosis as a function of clinical manifestations and PID categories (Fig 1, D and E). Some clinical manifestations had a higher chance of underlying a genetic disorder. Nevertheless, given the number of patients, the association was statistically significant only for mucocutaneous disorders characterized by infections or immunopathology. In contrast, the diagnostic rate was under 20% in the patients with neoplasia or neurological disorders (Fig 1, D). As expected, patients classified in the primary antibody deficiency category was the group with the least diagnostic rate (18%) (Fig 1, E).<sup>6</sup> Patients with or without previous genetic explorations had similar diagnosis yield with NGS procedure: 32% (n = 20 of 61) versus 29% (n = 20 of 68), respectively.

Our in-house tool for CNV identification displays a normalized ratio of coverage, gene-by-gene, for each patient, on a user-friendly graphic interface.<sup>7</sup> This tool rapidly detected CNV in 6 patients (15% of the positive cases) at homozygous state (n = 1) or heterozygous state (n = 5) (Table II). The size of the identified CNVs ranged from heterozygous deletion of 1 99-bp exon in *LYST* to a large heterozygous deletion of *TBX1* revealing a 22q11.2 deletion syndrome. One of these CNVs was a heterozygous duplication in *DOCK8* (duplication of exons 15 to 26) occurring with another heterozygous nonsense mutation (Table II). Multiplex ligation-dependent probe amplification analysis of sorted T cells showed that almost all circulating T cells have undergone somatic reversion of the duplication. Nevertheless, we observed a significantly increased duplication/deletion score when DNA from whole blood was analyzed. Another patient with combined immunodeficiency carried a hemizygous



**FIG 1.** **A**, Distribution of zygosity for the patients for whom mutations were identified in the PIDs cohort ( $n = 40$  patients). **B**, Distribution of the types of mutation found in the PIDs cohort ( $n = 46$  mutations). **C**, Diagnostic rate as a function of age. **D**, Evaluation of the diagnostic rate according to clinical features. Patients were described as having mucocutaneous, hepatic, digestive, pulmonary, or neurological disorders, whatever the type of sign (eg, infection, lymphoproliferation, atopy, neoplasia). Autoimmune manifestations other than cytopenia (50% of diagnoses,  $n = 18$ ), mucocutaneous disorders (47% of diagnoses,  $n = 46$ ), and clinical lymphoproliferation/organo-megaly (41% of diagnoses,  $n = 37$ ) were more frequently associated with genetic findings. **E**, Evaluation of the diagnostic rate according to PIDs categories. **C-E**, The curves represent the diagnostic rates. The histograms represent **(C)** the age distribution of the PIDs cohort (categories) and **(D and E)** the numbers of patients within the PIDs category concerned. CID, Combined immunodeficiency; PAD, primary immunodeficiency.

TABLE II. CNV analysis in the PIDs cohort

Patient	Gene	CNV	Dup/del score	Confirmation	Other mutation in the gene
PID10	<i>LRBA</i>	Homozygous deletion exons 30 to 34	0.01 to 0.02	Absence of PCR products and protein	None
PID54	<i>DOCK8</i>	Heterozygous duplication exons 15 to 26	1.28 to 1.52	MLPA	p.R1763*
PID74	<i>DOCK8</i>	Heterozygous deletion exons 1 to 26	0.42 to 0.56	MLPA	p.R550*
PID125	<i>TBX1</i>	Heterozygous deletion of the entire gene	0.48 to 0.61	FISH 22q11	None
PID126	<i>IGHM</i>	Heterozygous deletion of the entire gene	0.49 to 0.55	aCGH (hg37 : 106283841_107258824)	p.Q316*
PID127	<i>LYST</i>	Heterozygous deletion exon 48	0.51	Not confirmed genetically. Presence of neutrophil granules and hair abnormalities.	p.Q1068*

aCGH, Array comparative genomic hybridization; del, deletion; dup, duplication; FISH, fluorescent in situ hybridization; MLPA, multiplex ligation-dependent probe amplification.

nonsense mutation of *IL2RG* (c.982C>T; p.R328\*). However, this variant localized in the last exon is predicted to escape nonsense-mediated mRNA decay. Interestingly, 14% of the reads detected were wild type (51 of 363), suggesting a somatic reversion event.

Targeted NGS is a cost-effective supervised approach for the diagnosis of PIDs. Numerous previous studies have proven the efficacy of both targeted NGS and WES for diagnosis of PIDs (see Table E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).<sup>4</sup> We achieved a rate of diagnosis that is equal to the median of those previously published (31%). However, this rate is highly dependent on the population studied and the inclusion criteria. We chose to reach a relatively high depth of coverage, allowing the detection of somatic reversion events. These high-quality data, combined with a robust algorithm comparing coverage between patients, made it easy to identify CNVs rapidly and correctly. A few other groups also performed CNV analysis leading to a mean increase of diagnostic yield by 4.2%, comparable to the 4.6% increase in the current study.<sup>4</sup> The ethical concerns raised by incidental findings could be avoided by sequencing only PID-related genes.<sup>8</sup> Moreover, targeted NGS is easier to customize, with the possibility of designing probes for highly conserved intronic regions or untranslated regions. However, with dozens of new PID genes discovered each year, the targeted panels will need to be updated regularly. With this caveat, and given the continually decreasing costs of sequencing, WES or whole genome sequencing will probably become the new first-line strategy in a few years' time.<sup>9</sup> In conclusion, this study shows that using rapid, precise, and wide targeted NGS strategy has a clear beneficial impact for molecular diagnosis in PID patients.

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