Articles from the Special Issue on Genetic Errors of Immunity

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Consensus of the Italian Primary Immunodeficiency Network on the use and interpretation of genetic testing for diagnosing inborn errors of immunity

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Background: Inborn errors of immunity (IEIs) comprise more than 500 different rare congenital disorders of the immune system and are characterized by susceptibility to infection and immune dysregulation. The significant overlap of the clinical features among the different forms may lead to diagnostic delay. High-throughput sequencing techniques may allow a timely genetic definition. Guidelines for the use and the interpretation of genetic testing produced by the American College of Medical Genetics and Genomics (ACMG) and the European Society of Human Genetics (ESHG) do not cover specifics for their application to IEIs.
Objective: The aim of this consensus study was to define the best approach to genetic testing for IEIs.
Methods: A panel of experts in the context of the Italian Primary Immunodeficiency Network (IPINet) composed a list of statements that were evaluated by the Delphi method.
Results: The experts recommend that genetic testing for IEIs should be offered to selected patients with warning signs for IEIs and highlight the crucial role of thorough phenotyping and functional tests for the conclusive diagnosis of IEI. Comprehensive educational programs targeted to health care professionals and the public should be developed to increase IEIs awareness and reduce diagnostic delay. Ethical issues should be pondered over the diagnostic advantages of genetic tests requested for diagnostic purposes.
Conclusion: Adherence to guidelines on the use and interpretation of genetic tests for diagnosing IEIs should help limit the inappropriate use of these techniques, thereby reducing the risk of misdiagnosis and patient apprehension regarding inconclusive genetic results. (J Allergy Clin Immunol 2025;155:1149-60.)

Key words: *Inborn errors of immunity, consensus, genetic tests, high-throughput sequencing, whole-exome sequencing, next-generation sequencing, Sanger sequencing*

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Abbreviations used

ACMG: American College of Medical Genetics
BTK: Bruton tyrosine kinase
CMA: Chromosomal microarray
CNV: Copy number variation
ESHG: European Society of Human Genetics
GT: Gene therapy
GUS: Genes of uncertain significance
HSCT: Hematopoietic stem cell transplantation
HTS: High-throughput sequencing
IEIs: Inborn errors of immunity
IKBKG: Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma
IPINet: Italian Primary Immunodeficiency Network
IUIS: International Union of Immunological Societies
KREC: K-deleting recombination excision circle
NBS: Newborn screening
NBT: Nitroblue tetrazolium test
NCF1: Neutrophil cytosolic factor 1
NGS: Next-generation sequencing
SCID: Severe combined immunodeficiency
SIGU: Società Italiana di Genetica Umana
SS: Sanger sequencing
T-NGS: Targeted next-generation sequencing
TMS: Tandem mass spectrometry
TREC: T-cell receptor excision circle
VUS: Variants of unknown significance
WAS: Wiskott-Aldrich syndrome
WES: Whole-exome sequencing
WGS: Whole-genome sequencing
XHIGM: X-linked hyper-IgM syndrome

Inborn errors of immunity (IEIs) are rare inherited disorders of the immune system characterized by increased risk of infection, malignancy, and immune dysregulation.[1-4] Early genetic diagnosis of IEIs is crucial to promptly establish a specific treatment, prevent complications, and improve their outcome. Moreover, the genetic definition may allow accurate genetic counseling, reproductive advice, and identification of candidates for gene-specific therapies.[3,5] More than 500 IEI-causing genes have been identified to date. As a result of the phenotypic overlap among different IEIs, the diagnosis based on the clinical and/or immunologic phenotype remains often difficult, leading to a delay between the onset of symptoms and the diagnosis.[6,7] In the last few years, the introduction of high-throughput sequencing (HTS) techniques has expedited the identification of relevant genes in different monogenic conditions.[3,6] HTS represents a cost-effective genetic approach and has recently assumed the role of rapid first-tier test for the evaluation of complex cases of IEI.[8,9] The advantage of this technique is the simultaneous sequencing of gene panels, which might allow clinicians to rapidly identify an affected gene, including those that probably would not be sought using the traditional approach based on a functional-driven hypothesis. Indeed, the success of HTS in the field of IEIs is proven by the fact that since its first introduction, hundreds of novel gene defects responsible for IEI disorders have been identified, and novel clinical phenotypes associated with well-known disease-causing genes have expanded.[3,6] However, in spite of its numerous advantages, HTS usually reveals a great number of variants in patients as well as in the general population, making identification of disease-causing variants challenging.[10,11] Indeed, what we are finding is that human genetic diversity is much greater than that observed so far.

Functional tests are mandatory to confirm the pathogenetic role of variants of unknown significance (VUS) because a false assignments of pathogenicity can result in incorrect prognostic, therapeutic, or reproductive advice and in misallocation of resources for basic and therapeutic research.[12] Furthermore, HTS allows identification of the gene responsible for IEI in up to 40% to 60% of cases, depending on cohort characteristics, sequence technology, and analyses.[13-15] Within this framework, the identification of the genetic diagnosis may be hindered by technical and analytic issues, including the inability of HTS to identify large deletions or insertions, the presence of pseudogenes, or the fact that exome sequencing has largely focused on International Union of Immunological Societies (IUIS) genes, not considering that some molecular diagnoses could fell outside of this category, with novel genes implicated in the pathogenesis.[16] In addition, in specific IEIs (ie, IgA deficiency, common variable immunodeficiency), the pathogenesis is not understood except for a few monogenic cases.[17,18]

Nowadays, genetic tests may be more widely available in nonspecialized centers. Thus, immunologist advice is the cornerstone when an IEI disorder is suspected. In fact, the diagnosis of IEI may sometimes be supported by validated and reliable immunologic nongenetic tests sufficient to confirm the diagnosis and to drive therapeutic choice while awaiting genetic confirmation, or even in the absence of genetic confirmation.[19] Moreover, the use of HTS in nonspecialized settings, with lack of specific expertise and limited access to functional confirmation tests, may increase the risk of a false pathogenic ascription of a VUS, leading to misdiagnosis. Conversely, it should be noted that noninformative HTS analysis should not exclude an IEI, and these patients require strict monitoring and reassessment in a specialty setting. These observations raised the concern that, especially in nonspecialized settings, these tests might be inappropriately or prematurely used. In this context, the aim of this consensus is to provide recommendations on what and when to use IEI genetic testing and how to best interpret the results.

METHODS

This consensus study was performed using the Delphi method. In particular, a panel including experts in the fields of immunology, pediatrics, and genetics was appointed in the context of the IPINet. The panel included 4 clinicians with documented experience in the fields of immunology and pediatrics, and 4 molecular biologists with documented experience in the genetic diagnosis of IEIs and other nonimmunologic conditions. An extensive literature search through Medline was performed by using the following keywords: “genetic testing” AND “primary immunodeficiency” OR “inborn errors of immunity.” The literature regarding the use of HTS in various other genetic disorders was reviewed as well, and American College of Medical Genetics and Genomics (ACMG), European Society of Human Genetics (ESHG), and Società Italiana di Genetica Umana (SIGU) guidelines were carefully reviewed.[20] On this basis, as well as personal experience, the panel compiled a list of statements. Each statement was based on evidence drawn from (1) studies involving cases and case series of patients with IEIs, (2) use of HTS in

the diagnosis of other rare diseases, (3) rules of good practice derived from expert-based opinions, and (4) review of the literature from databases such as PubMed and Google Scholar. Scientific evidence came first, over clinical anecdotal experience. The statements were developed in response to the following key points:

- Optimal practices for IEI genetic testing (setting, timing, and the practices of education, consent, and counseling).
- Advantages, limitations, and appropriateness of genetic testing—including Sanger sequencing (SS), targeted next-generation sequencing (T-NGS), Mendeliome, whole-exome sequencing (WES)/whole-genome sequencing (WGS), and chromosomal microarray (CMA)—that is based on the clinical grounds of:
    - Limitations of HTS and the importance of its integration with traditional diagnostic approaches over the exclusive use of HTS.
    - Good practice to identify the actual role of VUS in affected genes.
    - Recommendations on how to manage patients with pathogenic genetic variants with incomplete penetrance or VUS.

A panel of 22 IPINet experts was appointed to rate the statements. At each round of evaluation, the statements had been submitted to the 22 panelists for rating. All the ratings received by the deadline were used to calculate the scores. Because biased answers represent a common risk for consensus documents, as a countermeasure, we asked panelists to provide response justification during all rounds. After each round, the statements were modified according to the panelists' comments and rated again in a subsequent round. The first round was rated by 19 of 22, the second by 19 of 22, the third by 17 of 22, and the fourth by 19 of 22. On the basis of the scores obtained, recommendations were rated from -1 (total disagreement) to $+1$ (total agreement). Agreement was defined as the sum of the ratings of strongly agree (rated $+1$) and agree (rated $+0.5$). Disagreement was defined as the sum of the ratings of strongly disagree (rated -1) and disagree (rated -0.5). If at least 75% of the raters agreed with the statement and a mean score of 0.75 or higher was reached, then that specific recommendation was assumed to have reached consensus. Furthermore, statements that reached a 75% level of agreement and a mean score between 0.65 and 0.74 were assumed to have reached only partial consensus. No consensus was defined as a mean score lower than 0.64 and agreement by less than 75% of the raters. According to Delphi methodology, the consensus was approved after the third round of opinions.

RESULTS

Develop optimal practices for IEI genetic testing

Setting and timing. Most raters agreed with the statement that the identification of warning signs of IEI should prompt an immunology consultation before genetic testing (rate of agreement, 100%) (Table I) to relieve the diagnostic journey and to accelerate the diagnosis for appropriate treatments. Indeed, definitive genetic diagnosis is based on the integration of the genetic information with clinical and laboratory features, which in turn require the expertise of specialists.[21] Moreover, evidence from the literature shows that a percentage of IEIs patients ranging from 30% to 85% (average, 62%) does not receive any genetic diagnosis through HTS.[13-15] Because IEI diagnosis may be based on clinical and laboratory diagnostic criteria even in the presence of noninformative genetic testing, an early IEI consultation is advised for the most appropriate treatment. Of note, in many clinically and immunologically defined cases (ie, common variable immunodeficiency, transient hypogammaglobulinemia of infancy, selective IgA deficiency), the etiology is likely to be nonmonogenic, and in some cases, the condition may represent a phenocopy of a genetically defined IEI.[17,18,22]

Practices of education. Most raters agreed with the statement that comprehensive educational programs targeted to health care professionals and the public should be developed to increase awareness of the IEIs' novel warning signs (rate of agreement, 100%) (Table I). In fact, paradigms of IEIs have expanded over the last few years, and apart from the classic warning signs of IEIs, the new ones are often unrecognized.[23,24] In particular, IEIs have traditionally been associated with an increased risk of severe, persistent, unusual, or recurrent infections. In the last few years, it has become clear that other noninfectious manifestations may be observed in IEIs patients, and may sometimes even represent the presenting sign.[2] These manifestations include autoimmune cytopenia, nonmalignant lymphoproliferation, severe eczema or erythroderma, autoimmune endocrinopathy, enteropathy, and rheumatologic manifestations, including vasculitis and systemic lupus erythematosus. For this reason, the patient may be initially seen by a specialist different from the immunologist. Moreover, the sensitivity of the traditional 10 warning signs for the diagnosis of IEIs is around 60% to 70%, and even lower for less severe phenotypes.[25] Because the patient is often assessed for the first time by specialists in different areas (ie, rheumatology, gastroenterology, hematology, dermatology, allergology) it is crucial to widen the awareness of these disorders among different specialties.[26-29]

Use of genetic testing for large-scale, population-based screening for IEIs. The raters did not agree with the statement that genetic testing should be used for large-scale, population-based screening (rate of agreement, 11%; mean score, -0.47 ± 0.44) (Table I), while the majority of raters agreed with the statement that population-based newborn screening (NBS) programs for IEIs based on T-cell receptor excision circles (TRECs)/K-deleting recombination excision circles (KRECs) and tandem mass spectrometry (TMS) should be implemented to increase the early identification of IEIs (rate of agreement, 100%) (Table I). Severe combined immunodeficiency (SCID) is the most severe form of IEI that leads to death in the first 2 years of life unless it is promptly diagnosed and treated.[30] The introduction of NBS based on TRECs/KRECs and TMS has been shown to dramatically anticipate the diagnosis, allowing timely interventions for affected infants.[30] The incidence of SCID went from 1:200,000 to 1:58,000 live births,[30] and may be even lower when the NBS is performed in populations with high consanguinity rates.[31,32] The early diagnosis of SCID has significantly improved the outcome of allogeneic hematopoietic stem cell transplantation (HSCT), with survival over 90% when the transplantation is performed in asymptomatic infants before 3.5 months of age, or of gene therapy (GT) when available.[3,33-36] NBS based on TRECs/KRECs also allows the early identification of other immune defects characterized by early-onset T- and B-cell lymphopenia.[37]

Some studies suggest that in addition to screening for SCID, which is already active in several countries,[38] other clinically

TABLE I. Setting, timing, and practices of education, consent, and counseling

Statement	Level of agreement (%)	Level of disagreement (%)	Neutral (%)	Score (mean ± SD)
Consensus				
Identification of warning signs should prompt immunology consultation before genetic testing.	100	0	0	0.95 ± 0.16
Comprehensive educational programs targeted to health care professionals and the public should be developed to increase the awareness of the warning signs of IEI.	100	0	0	0.92 ± 0.19
Genetic testing should be used for large-scale, population-based screening.	11	78	11	-0.47 ± 0.45
HTS-based population screening should be considered in the future for the following conditions:				
IEIs with available potentially curative therapies (allogeneic HSCT, GT), such as SCID, selected combined immunodeficiency diseases, and chronic granulomatous disease.	95	0	5	0.79 ± 0.30
Other conditions selected according to the specific populations to be screened (ie, founder effect or consanguineous populations).	53	21	26	0.18 ± 0.65
HTS-based NBS should supplement more than replace the current NBS system because it may show lower sensitivity and specificity rates compared to current TRECs/KRECs and TMS techniques.	79	10.5	10.5	0.63 ± 0.66
Population-based NBS programs for IEIs based on TRECs/KRECs and TMS should be implemented to increase early identification of IEIs.	100	0	0	0.87 ± 0.28
Before performing any kind of genetic testing, the patient should be adequately informed of the possible outcomes of HTS.	100	0	0	0.97 ± 0.11
Patients should be informed of the risk of:				
Inconclusive genetic results (VUS or variants in GUS).	100	0	0	0.95 ± 0.16
Identifying unsolicited findings.	100	0	0	0.79 ± 0.25
Identifying pathogenic mutations in IEIs with incomplete penetrance or in nonactionable genes.	95	0	5	0.79 ± 0.30
Genetic results can be delivered directly to the patient.	21	63	16	−0.42 ± 0.61
The patients should be offered the choice to opt in or out of the disclosure of being affected by nonactionable genetic conditions.	80	0	20	0.67 ± 0.40
It should be specified when analysis is limited to a list of specific immune genes, and analysis of secondary findings, as suggested by ACMG, is not performed.	88	6	6	0.76 ± 0.43
Partial consensus				
The patient should be informed of the risk of identifying the status of an asymptomatic carrier.	70	0	30	0.62 ± 0.45
No consensus				
The reporting of the carriage status for IEIs should be limited to X-linked conditions.	47	53	0	0.06 ± 0.88
Considering the rarity of the conditions, reporting pathogenic heterozygous variants of autosomal-recessive conditions should be avoided.	53	41	6	0.15 ± 0.86
Heterozygous variants of autosomal-recessive conditions should be reported only in specific cases, including those with a high degree of consanguinity or founder effect in specific genetic isolates.	35	53	12	0.20 ± 0.87

significant IEIs should be screened for at birth.[39,40] The raters agreed that HTS-based population screening should be considered in the future for IEIs with available potentially curative therapies (allogeneic HSCT, GT), such as SCID, selected combined immunodeficiency diseases, and chronic granulomatous disease (rate of agreement, 95%), as suggested recently by King et al,[39] although they did not agree that other conditions should selected on the basis of the specific populations to be screened (ie, founder effect or consanguineous populations) (rate of agreement, 53%) because the choice of the condition to be screened should be based on Wilson and Jungner criteria. The HTS-based approach is considered more suitable and reliable as a result of the significant heterogeneity of the conditions. However, although SCID fulfills Wilson and Jungner's 10 criteria,[40] there are concerns that other forms of IEIs, which could also be detected by an HTS-based NBS, may not fulfill these criteria.[38] Moreover, even though NBS based on HTS is an attractive modality in the field of IEIs,[41,42] it has limitations that may hinder its clinical application. One of the main limitations is represented by the uncertainty of the results, when VUS in known genes or genes of uncertain significance (GUS) are identified, and by the risk of identifying unsolicited findings and pathogenic mutations in IEIs with an incomplete penetrance (ie, CTLA4 [cytotoxic T-lymphocyte–associated antigen 4] haploinsufficiency, activated phosphoinositide 3-kinase δ syndrome, and others).[43,44] Moreover, IEIs include many nonactionable disorders (ie, ataxia telangiectasia and severe

forms of dyskeratosis congenita), the early diagnosis of which would not significantly improve a natural course characterized by an unrelenting progression of symptoms and reduced life expectancy. However, it should be noted that having these diagnoses (ie, ataxia telangiectasia) at an early age could help with prognosis, setting expectations, getting appropriate resources and specialists involved, and improving family understanding. Moreover, although the suggested diseases may not be cured, there are certainly "actions" (both immunologic and nonimmunologic) that may be of benefit, such as avoidance of live vaccines, especially rubella, in ataxia telangiectasia with significant T lymphocytopenia. Finally, there is the risk of identification of asymptomatic carriers. Although this could help in planning future pregnancies, the knowledge of a carrier status can cause anxiety that may affect reproductive choices.[45-47] It should be noted that HTS-based NBS may show lower sensitivity and specificity rates compared to current TRECs/KRECs and TMS techniques.[48] Thus, the raters partially agreed that HTS-based population screening should supplement more than replace the current NBS system (rate of agreement, 79%; mean score, 0.63 ± 0.66).

Ethical aspects. Most raters agreed with the statement that before performing any genetic testing, the patient (or parents/guardians in case of minors) should be adequately informed of the possible outcomes of HTS (rate of agreement, 100%) (Table I). This is in keeping with ACMG[20,49] and ESHG guidelines.[50,51] In particular, the raters agreed that they should be informed of the risk of inconclusive genetic results (VUS or variants in GUS) (rate of agreement, 100%), as well as of identifying unsolicited findings (rate of agreement, 100%) and pathogenic mutations in IEIs with incomplete penetrance or in nonactionable genes (rate of agreement, 95%). The raters did not agree that the patient should be informed of the risk of identifying a status of asymptomatic carrier (rate of agreement, 70%) (Table I). It was suggested by a few panelists that the reporting of the carriage status for IEIs should be limited to X-linked conditions in which there is a 50% risk for female carriers of having affected baby boys or carrier baby girls; and that considering the rarity of the conditions, reporting pathogenic heterozygous variants of autosomal-recessive conditions should be avoided because it could lead to an overdiagnosis and cause preconceptional anxiety and overtesting. Moreover, a few panelists proposed that heterozygous variants of autosomal-recessive conditions should be reported only in specific cases, including those with a high degree of consanguinity or founder effect in specific genetic isolates. However, the raters did not agree on these statements (rate of agreement, 47%, 53%, and 35%, respectively) (Table I). In fact, some of them raised the concern that the reporting of a heterozygous variant associated with a clinical phenotype of an autosomal-recessive IEI should prompt more genetic testing to identify a second variant on the other allele. The raters partially agreed that in view of patients' autonomy and their right (not) to know, patients should be offered the choice to opt in or opt out of the disclosure of being affected by nonactionable genetic conditions (rate of agreement, 80%; mean score, 0.67 ± 0.40) (Table I). The opt-in/opt-out choice should be pursued at the bioinformatic analysis stage, so if the patient opts out, unsolicited findings should not be included in the final report. The raters raised the concern that it could be difficult to define medical actionability in the field of IEIs where therapeutic options are rapidly emerging. This could pose ethical dilemmas because novel therapeutic options for nonactionable conditions may be available in the near future. Moreover, nonactionable conditions could benefit from specific follow-up and support therapy as well as periodic examinations based on a specific genetic diagnosis.

Even though unsolicited findings are identified in less than 1% of patients,[52] such findings may have a significant impact on the single patient. According to ACMG guidelines, the genetic report should include secondary findings, defined as actively sought known pathogenic or expected pathogenic variants in a defined set of genes that are considered medically actionable, even when unrelated to the primary medical reason for testing.[49,53,54] The list of medically actionable genes is updated and refined annually by the ACMG Secondary Findings Working Group and Board of Directors.[55] It should be noted that this list mainly includes genes related to cancer and cardiovascular phenotypes.[55] The question of how to define medical actionability represents a difficult matter that should be carefully evaluated with experts in the field. Thus, the formation of committees in the context of immunology societies should be promoted to define a list of genes associated with medically actionable IEIs and to avoid ambiguity. However, it should be noted that in some cases, genetic testing is performed by facilities that have specific expertise in IEIs. Thus, the raters agreed that in these cases, it should be specified that the analysis is limited to a list of specific immune genes and that the analysis of secondary findings, as suggested by ACMG, is not performed (rate of agreement, 88%) (Table I). It should be also considered that genetic tests in IEIs are often requested in pediatric patients. Thus, as a result of their inability to make autonomous choices, the health care provider should pay particular attention in disclosing information that may affect the child's right (not) to know, especially when the decision could be postponed until the child could decide for him- or herself.[46]

Finally, the raters did not agree that genetic results can be delivered directly to the patient (rate of agreement, 21%) (Table I). They suggest that the genetic results should be discussed with the clinician ordering the test in the context of a genetics/immunology consultation. In fact, if not properly discussed, the result of the genetic test might lead to disproportionate reactions and excessive anxiety for benign or inconclusive results.[56] The ACMG clearly indicates that clinicians have the responsibility to provide comprehensive counseling to all patients before and after undergoing HTS analysis.[20,49] If the clinician ordering the test does not feel confident in providing complete information at any stage of the pre- and/or postcounseling process, then the patient should be referred to an expert in the field, as is also suggested by the ACMG.[20]

Develop guidelines on the appropriateness of genetic testing based on the clinical setting

Today, many different genetic testing methods are available, all with advantages and limitations.[57] It is not possible to define a specific algorithm for diagnosis because the choice of genetic testing depends on access to resources at a single center. However, guidelines on the appropriateness of the type of genetic testing —based on clinical setting and diagnostic hypothesis—should be developed. Because of the potential physical and/or emotional implications of the identification of unsolicited findings, the ESHG and the Canadian College of Medical Genetics recommend targeted approaches to sequencing to minimize the

likelihood of identifying unsolicited findings.[51,58-60] However, even though the reporting of a VUS completely unrelated to the clinical phenotype should be limited, larger panels should not be seen as a threat to incidental findings. In fact, incidental findings may be sometimes be the key to a diagnosis, by uncovering genetic nonimmunologic conditions that may present as IEIs. Thus, in case the diagnosis has not been identified with narrow panels, the analysis should be extended to larger panels to increase the likelihood of reaching a definitive genetic diagnosis.

SS. Compared to NGS technology, in specific clinical settings, SS may be faster, less expensive, and more reliable, with no risk of unsolicited findings. The recent study of Chan et al[61] suggested, on the basis of the high sensitivity and accuracy, fast turnaround time, low cost, and fewer VUS and no secondary findings,[13,19] that single specific targeted gene SS should remain the first-tier genetic test for patients suspected to have 1 of the 5 common X-linked IEIs (X-linked agammaglobulinemia, Wiskott-Aldrich syndrome [WAS], X-linked chronic granulomatous disease, X-linked SCID, and X-linked hyper-IgM syndrome [XHIGM]). The raters agreed that SS should be preferred in the presence of an affected family member with a known mutation (rate of agreement, 100%), for the confirmation of prenatal diagnosis (rate of agreement, 100%), for the study of segregation (rate of agreement, 95%), and when the diagnosis is suggested by a positive functional test and the suspected disease is caused by a small gene (up to 20 exons) traditionally studied through SS (ie, *ADA, PRF1, SH2D1A, WAS, CYBB*) or characterized by hot spot or known founder mutations (rate of agreement, 89%) (Table II). There was no consensus on the statement that SS should remain the first-choice genetic test for patients suspected to have one of those IEIs easily studied with SS (rate of agreement, 54%) (Table II). Finally, the raters partially agreed that SS should be used for the confirmation of a variant identified through NGS technology (rate of agreement, 79%; mean score, 0.68 ± 0.48) (Table II). The SIGU recommends that all the variants reported should be confirmed on an independent sample using a different technique (ie, SS).[62,63] The confirmation can be avoided in case the examination is performed via trio exome analysis and when the coverage for the proband is deemed adequate (>40×), although it is strongly suggested for all *de novo* variants.[62,63]

Targeted NGS. NGS technology offers the advantage of a simultaneous analysis of many genes. On the one hand, the larger the panel, the higher are the odds of identifying VUS or unsolicited findings that, on the other hand, may sometimes represent the key to the diagnosis.[10,52] The raters partially agreed that in clinical practice, smaller, targeted-IEI NGS panels may be useful only if genes are clustered for specific phenotypes and if patient symptoms match a specific type of IEI (rate of agreement, 89%; mean score, 0.66 ± 0.34), while they agreed that for complex/atypical phenotypes, the largest available panels (Mendeliome, WES, WGS)[11] should be preferred (rate of agreement, 100%) (Table II). In fact, the extra cost of applying multiple smaller panels could be a disadvantage in complex phenotypes.[11] The raters agreed that the list of genes analyzed in T-NGS should be always made available to the patient for future consultations or second opinions (rate of agreement, 95%), and that before requesting a specific panel, the clinician should check for the presence of genes that may explain the clinical phenotype observed in the patient (rate of agreement, 89%) (Table II). The raters partially agreed that in the analytic phase, the sequenced genes could be filtered by clinical priorities to limit the identification of unsolicited findings (rate of agreement, 90%; mean score, 0.70 ± 0.34) (Table II). The genetic report should include only the list of the candidate genes associated with the diagnostic question.[55,59,62] However, the most common tools of gene-variant analysis offer the possibility of identifying novel disease candidate genes that could be added to the final list, following the indications provided by the ACMG and ESHG about the disclosure of unsolicited/secondary findings.[20,59] No consensus was found on the size of the genetic panel for targeted NGS. However, the highest agreement was found for the largest suggested panel size, including all the known IEIs genes (rate of agreement, 79%; mean score, 0.58 ± 0.58) (Table II).

Mendeliome and WES. Mendeliome and WES analyses offer the advantage of identifying mutations in a reasonable amount of time in all disease-causing genes or in the whole exome. There is a high risk of identifying VUS, variants in GUS, or unsolicited findings. They can be expensive and time-consuming. The raters agreed that these tests should be preferred when the condition could be caused by nonimmune or novel genes that may not be included in the T-NGS panels (rate of agreement, 89%) (Table II). Moreover, the raters agreed that trio exome analysis may allow the identification of novel disease-causing genes and novel phenotypes associated with known genes (rate of agreement, 100%) (Table II). Novel disease-causing genes are continuously identified, especially in the field of IEIs. Recent evidence suggests that reanalysis of WES is more useful than performing a new analysis through WGS.[64,65] For this reason, Mendeliome/WES should be reanalyzed over time in light of novel acquisitions in the field. However, the raters did not agree that the analysis should be systematically repeated in all patients every other year (ie, the timing of the publication of the IUIS classification) (rate of agreement, 42%), while they agreed that it should be requested by the clinician in selected cases on the basis of clinical features and on acquisitions from the literature (rate of agreement, 95%) (Table II).

CMA. IEI may be caused by structural genetic changes that are not easily detected through HTS. The term *structural variant* refers to alterations larger than 50 nucleotides[66] and includes insertions, deletions, duplications, inversions, and translocations. Deletions and duplications are also defined as copy number variations (CNVs) and account for 5% to 10% of all deleterious genetic effects.[67] CMA, including multiplex microarray-based comparative genomic hybridization and single nucleotide polymorphism microarrays,[68] ligation-dependent probe amplification,[69] and fluorescence *in situ* hybridization can be used to detect CNVs, including microdeletions, microduplications, and translocations, and may significantly increase the diagnostic rate of HTS.[11,70-72] However, with the current quality of NGS data, it is also possible to detect some CNVs.[70,72-75] The raters agreed that CMA should be performed in the presence of syndromic features (intellectual disability, cardiovascular malformations) (rate of agreement, 100%), to evaluate the presence of structural genetic changes in patients with a suspected recessive disease where a heterozygous point mutation is detected in a good candidate gene (rate of agreement, 100%), when there is a strong suspicion of a specific IEI but the HTS could not identify any genetic alteration, especially when the suspected IEI is caused by large genes prone to deletion or rearrangement (ie, *NCF1* [neutrophil cytosolic factor 1], *DCLRE1C, CYBB, BTK, C1INH,* and *DOCK8*)[76-84] (rate of agreement, 89%) and in the suspicion of a dual diagnosis caused by a deletion and a point

TABLE II. Choice of type of genetic testing based on clinical setting

Statement	Level of agreement (%)	Level of disagreement (%)	Neutral (%)	Score (mean ± SD)
Compared to NGS technology, SS is faster, less expensive, and more reliable. There is no risk of incidental findings. It should be preferred in the following situations:				
Consensus				
Presence of affected family member with known mutation.	100	0	0	0.97 ± 0.11
Confirmation of prenatal diagnosis.	100	0	0	0.95 ± 0.16
Study of segregation.	95	0	5	0.92 ± 0.25
Diagnosis is suggested by positive functional test result and the suspected disease is caused by a small gene (up to 20 exons) traditionally studied through SS (ie, *ADA, PRF1, SH2D1A, BTK, WAS, CYBB*) or characterized by hot spot or known founder mutations.	89	0	11	0.76 ± 0.35
Partial consensus				
Routinely used confirmation of a variant identified through NGS technology.	79	5	16	0.68 ± 0.48
No consensus				
SS should remain the first choice for patients suspected to have one of the IEIs easily studied through SS (eg, *PRF1, SH2D1A*).	58	21	21	0.18 ± 0.71
NGS technology offers the advantage of a simultaneous analysis of a large number of genes. The larger the panel, the higher the odds of identifying VUS or incidental findings. Concerning targeted panels, the following statements were produced:				
Consensus				
In clinical practice, smaller, targeted IEI NGS panels may be useful if genes are clustered for specific phenotypes and patient symptoms match a specific type of IEI.	89	0	11	0.66 ± 0.34
For complex/atypical phenotypes, the largest available panels (including Mendeliome, WES, and WGS) should be preferred.	100	0	0	0.87 ± 0.23
The list of genes analyzed in T-NGS should be always made available to the patient.	95	5	0	0.84 ± 0.37
Before requesting a specific panel, the clinician should check for the presence of the genes that may explain the clinical phenotype observed.	89	5	5	0.89 ± 0.27
Partial consensus				
In the analytic phase, sequenced genes could be filtered by clinical priorities to limit identification of unsolicited findings.	90	0	10	0.70 ± 0.34
No consensus				
The optimal size of a T-NGS panel is:				
<50 genes	32	42	26	0.00 ± 0.53
50-100 genes	32	26	42	-0.05 ± 0.52
100-150 genes	32	11	58	0.11 ± 0.32
All the known IEI genes (400-450 genes)	79	11	11	0.58 ± 0.58
Mendeliome/WES analyses offer the advantage of identifying mutations in a reasonable amount of time in all the disease-causing genes or in the whole exome. There is a high risk of identifying VUS or incidental findings. They can be expensive and time-consuming. Mendeliome/WES should be preferred in the following situations:				
Consensus				
The condition could be caused by nonimmune or novel genes that may not be included in the T-NGS panels.	89	5	5	0.82 ± 0.42
Trio exome analysis may allow identification of novel disease-causing genes and novel phenotypes associated with already known genes.	100	0	0	0.87 ± 0.23
Novel disease-causing genes are continuously identified, especially in the field of IEIs. For this reason, Mendeliome should be reanalyzed in light of novel acquisitions in the field. The optimal timing for the reanalysis is:				
Consensus				
Reanalysis should be requested by the clinician in selected cases on the basis of clinical features and on data in the recent literature.	95	0	5	0.87 ± 0.21
No consensus				
Every other year (timing of publication of the IUIS classification).	42	37	21	0.03 ± 0.61
CMA offers the advantage of detecting CNVs, microdeletions, microduplications, and translocations. CMA should be performed in the following situations:				
Consensus				
In the presence of syndromic features (eg, intellectual disability, cardiovascular malformations).	100	0	0	0.82 ± 0.25
To look for the presence of structural genetic changes in patients with suspected recessive disease where a heterozygous point mutation is detected in a good candidate gene.	100	0	0	0.95 ± 0.16
Strong suspicion of a specific IEI, but the HTS could not identify any genetic alteration.	89	0	11	0.84 ± 0.34
Suspicion of a dual diagnosis caused by a deletion and a point mutation in an immune gene (eg, Williams syndrome with chronic granulomatous disease).	95	0	5	0.76 ± 0.31
The use of bioinformatic tools should be promoted to increase the diagnostic rate of HTS.	100	0	0	0.85 ± 0.24

TABLE III. Limitations of HTS and importance of traditional diagnostic approach over exclusive use of HTS

Statement	Level of agreement (%)	Level of disagreement (%)	Neutral (%)	Score (mean ± SD)
Nowadays, genetic tests are largely also available in nonspecialized centers. However, if an IEI is suspected, an immunologist should always be consulted. In fact, the diagnosis of IEI must be supported, when applicable, by validated and reliable functional tests that should be used to confirm the diagnosis and to drive therapeutic choices while awaiting genetic confirmation, or even in the absence of genetic confirmation. Genetic tests have limitations that may hinder the diagnosis of IEI. For example, the genetic diagnosis may be affected by the presence of pseudogenes (ie, *NCF1, IKBKG*) or by the difficulties in identifying large structural alterations or deep intronic mutations.				
Consensus				
The availability of high-throughput genetic tools should not limit the use of traditional approach to the diagnosis of IEI on the basis of clinical and laboratory criteria and on the use of specific immunologic tests.	89	0	11	0.87 ± 0.33
Immunologic tests, when available, should be used to confirm the diagnosis and drive therapeutic choices while awaiting genetic confirmation.	95	5	0	0.81 ± 0.38
The indication for the genetic testing and the type of genetic test should be given by an expert in immunology.	95	0	5	0.87 ± 0.28
A negative result of a targeted or WES panel does not exclude the diagnosis of IEI, which may be confirmed by other validated nongenetic tests.	95			
Additional tests, including long-read cDNA sequencing, WB, or transcriptomics, may be used to confirm the diagnosis at the molecular level in more complex cases.	89	0	11	0.82 ± 0.34
Immunologic tests should be performed in validated laboratories, and diagnosis requires the expertise of a clinical immunologist.	95	0	5	0.89 ± 0.27
Reliable tests to confirm the diagnosis of specific IEI include:				
TMS or enzymatic assay to confirm suspicion of ADA or PNP deficiency.	95	0	5	0.89 ± 0.27
Flow cytometry–based surface or intracellular protein expression for (for example) WASp, BTK, CD40L, SAP, and XIAP to support suspicion of Wiskott-Aldrich syndrome, X-linked agammaglobulinemia, XHIGM, and X-linked lymphoproliferative syndrome types 1 and 2, respectively.	100	0	0	0.95 ± 0.16
NBT and DHR to confirm suspicion of chronic granulomatous disease.	100	0	0	0.95 ± 0.16

ADA, Adenosine deaminase; *DHR,* dihydrorhodamine test; *PNP,* Purine nucleoside phosphorylase; *WB*, Western blot.

mutation in an immune gene (ie, Williams syndrome with chronic granulomatous disease) (rate of agreement, 95%) (Table II). Moreover, the raters agreed that the use of bioinformatic tools[70,72-75] should be promoted to increase the diagnostic rate of HTS (rate of agreement, 100%) (Table II).

Define the limitations of HTS and the importance of integration with traditional diagnostic approaches over the exclusive use of HTS

The raters agreed that the availability of high-throughput genetic tools should not limit the use of traditional approaches to the diagnosis of IEI based on clinical and laboratory criteria (ie, European Society for Immunodeficiencies criteria) and on the use of specific immunologic tests (ie, nitroblue tetrazolium [NBT] test, flow cytometry–based surface or intracellular protein expression) driven by the clinical phenotype (rate of agreement, 89%), and that immunologic tests, when available, should be used to confirm the diagnosis, so they can drive therapeutic choices while awaiting genetic confirmation, especially when the condition is life-threatening or when an accurate diagnosis can modify the therapeutic approach and outcome (rate of agreement, 95%) (Table III). It should also be considered that thorough clinical and laboratory phenotyping is crucial for the correct interpretation of the results of the genetic analysis.[21] In low-income countries with limited access to HTS, a high degree of consanguinity, and recurrent genetic mutations, immunologic tests have been proven to be rapid and highly effective in confirming the diagnosis at the molecular level.[19,85]

Genetic tests have limitations that may hinder the diagnosis of IEI.[57] For example, they may be affected by the presence of pseudogenes in specific IEI genes (ie, *NCF1* or *IKBKG* [inhibitor of nuclear factor kappa B kinase regulatory subunit gamma]) or by the difficulties in identifying structural alterations.[57] Thus, the raters agreed that the indication for the genetic testing and the type of genetic test should be given by an expert in immunology who knows the limitations of the test used for some specific conditions (rate of agreement, 95%), that a negative result of a targeted or a WES panel does not exclude the diagnosis of IEI that may be confirmed by other validated nongenetic tests (rate of agreement, 95%), and that because mutations in a number of IEI genes (ie, *NCF1, IKBKG, ATAD3A, CDC42, USP18*) may

TABLE IV. Recommendations on interpretation of VUS

Statement	Level of agreement (%)	Level of disagreement (%)	Neutral (%)	Score (mean ± SD)
Consensus				
The role of the variants identified in immune genes should be discussed with immunologist and/or geneticist with experience in the interpretation of HTS in IEIs.	95	5	0	0.82 ± 0.48
The creation of national and international disease-specific databases for data sharing should be encouraged to improve the definition of the role of specific variants.	100	0	0	0.84 ± 0.29
Geneticists and health care providers should be very careful in excluding or confirming a variant's pathogenicity solely on the basis of the congruence between the clinical features and the identified gene, without providing direct evidence of its pathogenic role, especially when the condition is not fully penetrant.	95	0	5	0.80 ± 0.30
No consensus				
A sample of the parents for the study of segregation should be always obtained in association with NGS (and also in cases of targeted panels).	79	5	16	0.66 ± 0.34

not be identified as a result of the presence of pseudogenes or homologous sequences, in the suspicion of these conditions, other tests, including long-read cDNA sequencing, Western blot analysis, or transcriptomics may be used to confirm the diagnosis at the molecular level (rate of agreement, 89%) (Table III).

Moreover, novel genes responsible for IEIs are continuously discovered.[3,6,86] Thus, in the presence of a diagnosis of IEI that is based on clinical and laboratory criteria, the immunologist can request additional genetic and nongenetic tests to confirm the diagnosis at the molecular level.[19] The ACMG guidelines support the use of functional tests as a powerful tool in the definition of pathogenesis.[20] As for other genetic conditions, also in IEIs, certain immunologic tests, including, for example NBT, dihydrorhodamine test, and TMS, offer well-established approaches to confirm or exclude the role of the variant identified.[19,87-92] In a few cases, an experimental approach may provide additional valuable information regarding the definition of the variant's role.[19] Validation, reproducibility, and robustness of the test are absolute requirements to confirm the pathogenicity. For this reason, the panel recommends that these tests be performed in validated laboratories and that an expert in clinical immunology is involved in the analytic process (rate of agreement, 95%) (Table III). The raters agreed that reliable tests to confirm the diagnosis of specific IEI include TMS or enzymatic assay to confirm a suspicion of adenosine deaminase deficiency or purine nucleoside phosphorylase deficiency (rate of agreement, 95%), NBT and dihydrorhodamine test to confirm the suspicion of chronic granulomatous disease (rate of agreement, 100%), flow cytometry–based surface or intracellular protein expression for (for example) WASp, Bruton tyrosine kinase (BTK), CD40L, SAP, and XIAP to support the suspicion of WAS, X-linked agammaglobulinemia, XHIGM, and X-linked lymphoproliferative syndrome types 1 and 2, respectively (rate of agreement, 100%) (Table III).[93-99]

Provide recommendations on interpretation of VUS

Inconclusive test results due to VUS represent a very common outcome of multigene panel analysis. In a recent article, the rate of inconclusive test results was 22.5% for WES/WGS and 32.6% for targeted gene panels.[10] As expected, the rate of inconclusive results correlated with panel size.[10] The authors suggest that the rate of VUS in targeted gene panels compared to WES/WGS may be higher despite the lower number of analyzed genes as a result of the practices of genetic testing laboratories of reporting all VUS when performing diagnostic targeted gene-panel testing.[10] The rationale is that the reported VUS may represent a hint for the clinician that, based on the provided diagnostic suspicion, may lead to the performance of additional testing that can help define the pathogenic role of the identified VUS. However, the clinicians receiving genetic reports should carefully evaluate the results, involving experts in the field and avoiding an overestimation of the role of variants that may only partially explain the phenotype.[11]

A pathogenic variant is defined as a variant that is deleterious or harmful, or that increases the probability of disease.[64,65,100] Specific criteria based on the population frequency, computational data (*in silico* prediction models), predictive data (eg, whether the variant affects important domains or causes loss of protein function), along with the functional and clinical data, have been outlined in joint guidelines from the ACMG and the Association for Molecular Pathology to define the pathogenicity of a variant.[20] Population databases may be used to define the frequencies of the variants in large populations and disease databases, and a literature search may help define the correlation between variant and clinical phenotype.[20,101] However, the exclusive use of disease databases for the assessment of the variants' pathogenicity may be limited by the fact that many databases may contain variants that are incorrectly classified, especially in databases that do not perform primary review of the evidence. Moreover, disease databases may not be promptly updated. For this reason, the ACMG guidelines recommend that laboratories collaborate with clinicians to allow a better definition of the pathogenicity of the variant identified.[20] The key importance for the teams to communicate should be emphasized. In fact, discussion of results between geneticists and immunologists may be crucial to clarify the clinical situation. Identification of a variant in an immune gene should prompt the immunologic consultation to define the likelihood of the diagnosis and drive, when available, immunologic assays to define the variant's role.[19,87-99] In fact, VUS reported in a disease manifesting with typical features of the disease should be taken more seriously than the ones where typical disease features are absent. Moreover, through national or international collaborations,

the patient may be referred to experts in the study of the specific disorder.[102] Indeed, the same variant identified in other patients or a different variant in the same GUS might help define the role of the variant or GUS in the observed clinical phenotype. Disease databases do not usually contain variants or GUS that are under evaluation. In most cases, the evaluation of the pathogenic role of a variant or a GUS takes many years. It should be also considered that negative results, excluding the pathogenic role of a variant, are often not reported in the literature. Thus, referral to an immunologist may allow access to unpublished data that may in turn expedite the pathologic definition. Moreover, immunologic consultation may help define the medical actionability of variants identified in IEI genes.

The raters agreed that the role of the variants identified in immune genes should be discussed with immunologists and/or geneticists with experience in the interpretation of HTS in IEIs (rate of agreement, 95%), and that the creation of national and international disease-specific databases for data sharing should be encouraged to improve the definition of the role of specific variants (rate of agreement, 100%) (Table IV).

The raters agreed that geneticists and health care providers should be very careful in excluding or confirming the pathogenicity of a variant only on the basis of the congruence between the clinical features and the identified gene, without providing direct evidence of its pathogenic role,[12,64,65] especially when the condition is not fully penetrant (rate of agreement, 95%) (Table IV). For example, the variant p.Leu297Val of the *TLR3* gene identified in a patient with adult-onset herpetic encephalitis[103] was shown to have no functional effect in peripheral blood mononuclear cells and fibroblasts obtained from 2 siblings with neonatal onset of herpetic encephalitis carrying the same variant (personal observation; unpublished data). It should be kept in mind that variants reported as pathogenic are likely to be considered actionable by health care providers, who may use the information to modify the treatment, surveillance plan, or family counseling.

Data from the literature suggest that the use of trio exome analysis significantly reduces the rates of inconclusive results.[10,11] However, the raters did not agree that a parental sample for the study of segregation should be always obtained in association with NGS and in cases of targeted gene panels (rate of agreement, 79%) (Table IV).

DISCUSSION

HTS techniques represent powerful tools to establish prompt diagnosis of IEIs and identify novel genes in appropriate specialty settings. However, they present limitations that need to be considered in clinical practice. Here we propose a differential approach to genetic diagnosis of IEIs that is based on phenotype complexity. In particular, we suggest focusing the analysis on narrow panels or single genes for well-defined clinical phenotypes to limit the identification of unsolicited and inconclusive findings. However, because incidental findings may uncover genetic non-immunologic conditions that may present as IEIs, the analysis should be extended to larger panels for more complex/atypical phenotypes or when the diagnosis has not been reached.

The inappropriate use of these techniques may carry a risk of misdiagnosis, which may prolong the diagnostic odyssey, lead to wrong therapeutic and reproductive advice, and cause apprehension regarding inconclusive genetic results. Thus, genetic testing for IEIs should be offered to selected patients with warning signs for IEI after thorough phenotyping and detailed immunologic evaluation. The immunologist should be in charge of such cases not only to provide the best interpretation of the genetic results but also to guide the management of the patient while awaiting genetic confirmation, or even in the absence of a genetic definition. Ethical issues should be pondered regarding the diagnostic advantages of genetic tests requested for diagnostic purposes.

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Key messages

- **Inappropriate use of genetic testing for the diagnosis of IEIs carries the risk of misdiagnosis that may prolong the diagnostic odyssey and lead to wrong therapeutic and reproductive advice.**
- **Our guidelines aim to guide clinicians in defining the best timing and setting for genetic testing and in warranting the best interpretation of results.**

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