State-of-the-Art Targeted High-Throughput Sequencing for Detecting Inherited Platelet Disorders

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Abstract

Inherited platelet disorders (IPDs) are a heterogeneous group of rare entities caused by molecular divergence in genes relevant for platelet formation and function. A rational diagnostic approach is necessary to counsel and treat patients with IPDs. With the introduction of high-throughput sequencing at the beginning of this millennium, a more accurate diagnosis of IPDs has become available. We discuss advantages and limitations of genetic testing, technical issues, and ethical aspects. Additionally, we provide information on the clinical significance of different classes of variants and how they are correctly reported.

Keywords

- blood platelet disorders
- high-throughput nucleotide sequencing
- platelets

Zusammenfassung

Angeborene Thrombozytenstörungen (IPDs) sind eine heterogene Gruppe seltener Krankheiten, die durch molekulare Divergenz in Genen verursacht werden, die für die Bildung und Funktion von Blutplättchen relevant sind. Ein rationaler diagnostischer Ansatz ist notwendig, um Patienten mit IPDs zu beraten und zu behandeln. Mit der Einführung der Hochdurchsatz-Sequenzierung zu Beginn dieses Jahrtausends ist eine präzisere Diagnose von IPDs möglich geworden. Wir diskutieren Vorteile und Einschränkungen von genetischen Tests, technische Probleme und ethische Aspekte. Zusätzlich bieten wir Informationen über die klinische Bedeutung verschiedener Klassen von Varianten und wie sie korrekt gemeldet werden.

Schlüsselwörter

- angeborene Thrombozytenstörung
- Hochdurchsatz-sequenzierung
- Thrombozyten
- Blutplättchen

Introduction

Platelets are main players in hemostasis. They do not only act as sentinels of the vascular integrity but can also seal wounds by forming a hemostatic plug, a process referred to as primary hemostasis.[1] In addition, platelets can build a procoagulant surface, where certain steps of the coagulation cascade take place thus bridging the primary with the secondary hemostasis.[2] Ectopic platelet activation, in contrast, can result in thrombotic events that eventually lead to myocardial infarction or stroke. The equilibrium of hemostasis and thrombosis has been the center of platelet research for decades, but it has been increasingly recognized that platelets also play an important role as immune mediators, where tissue- and context-specific

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roles become more clear, as indicated by thrombo-inflammation or immunothrombosis (like in COVID-19).[3,4] Platelets have an overall short half-life of 8 to 10 days before they become sequestered by the reticuloendothelial system. They are thus continuously replenished from megakaryocytes (MKs), the immediate precursor cell in the bone marrow, with $\sim 10^{11}$ generated per day.[5] Defects in platelet count or function are typically referred to as inherited platelet disorders (IPDs). They can be divided into (1) inherited thrombocytopenias, in which the paucity of platelets is a consequence of insufficient production (either amegakaryocytic or hypomegakaryocytic) or a shortened lifespan, and (2) inherited platelet function defects (PFDs) that are typically associated with an overall normal platelet count. The underlying causes of PFDs are dysfunctional surface receptors, defects in the signaling cascades, cytoskeletal alterations, inadequate number of internal granules, their contents, or an inappropriate release. Defects in MK-specific transcription factors can affect multiple of those mentioned aspects. IPDs can occur as an isolated feature or as a syndromic disorder with additional symptoms. Some IPDs have specific pathognomonic features such as Glanzmann's thrombasthenia (GT), Bernard-Soulier syndrome (BSS), or Congenital Amegakaryocytic Thrombocytopenia (CAMT) of which the relevant gene (or genes) has been well studied.[6–9] Other examples for IPDs that have been deciphered in the last two decades include Gray platelet syndrome[10] or thrombocytopenia-absent radius syndrome.[11,12] So far approximately 60 to 80 genes are considered to be involved in IPDs.[13] However, the genetic and experimental evidence for many of them may still be considered to be incomplete. International consortia are thus constantly evaluating the underlying evidence, which is finally reflected by a list of GoldVariants that are the "Tier 1" level of genes to be taken into account. New genes are considered, but genes are also removed when the evidence is insufficient.[5] The relevance of clinical complications in patients with IPDs is highly variable, even within the same disease type, ranging from almost negligible to life threatening. As some patients experience massive bleeding in response to surgery or trauma, it is important to know the underlying condition.[14] Consequently, an early and accurate diagnosis of the disorder and a close medical follow-up of the affected patient is of great relevance. Although research over the last three decades has led to the discovery of a relevant number of genes harboring variants responsible for IPDs,[13] evidence of pathogenicity of some of these genes remains limited. More importantly, more than a third of affected patients still do not receive a sound molecular (genetic) diagnosis.[15] In this article, we aim to elucidate the state-of-the-art high-throughput sequencing (HTS) in the diagnosis of IPDs, discuss advantages and disadvantages of this method, and describe challenges that have to be considered by those involved in the diagnosis and management of patients with IPDs.

Traditional Diagnosis of IPDs and Its Limitations

Several guidelines and expert committees recommend the following evaluations as first-line diagnostics of IPDs: (1) clinical investigation of the personal and familial bleeding history with an emphasis on unexplained or extensive bleeding events; (2) physical examination assessing for typical bleeding manifestations and for potential syndromic features (e.g., hearing loss, cardiac anomalies, facial dysmorphia or skeletal abnormalities, ocular involvement, skin discoloration, and mental retardation); (3) comprehensive laboratory testing for defects in secondary hemostasis; (4) exclusion of von Willebrand disease; and (5) evaluation of a complete blood cell count (CBC) and blood smear, especially focusing on platelet count, size (mean platelet volume [MPV]), and morphology (►Fig. 1).[16–18] In general, phenotypic and functional analyses usually start with relatively simple, widespread, and largely nonspecific tests (e.g., CBC, blood smear, prothrombin time, von Willebrand factor). Subsequently, more specific and complex methods (e.g., platelet aggregometry, flow cytometry analysis, immunofluorescence staining) are typically used.[19–22] However, the requirement of relatively large volumes of freshly drawn blood (especially for platelet function analyses) and the availability of these tests in a small number of highly specialized laboratories limit the speedy and sound diagnosis of IPDs, although much progress has been made regarding the standards and quality control of diagnostics in pediatric patients.[16,20,23]

Indications for Genetic Testing

A worldwide laboratory survey of the *International Society on Thrombosis and Haemostasis* (ISTH) revealed that even if a broad range of analytical methods is applied, the exact underlying defect cannot be identified in more than one-third of patients with confirmed abnormalities of platelet function.[24] However, a low platelet count does not exclude an associated altered platelet function; even if the functional defect is minor, it could contribute to the bleeding phenotype.[25] Indeed, a definite diagnosis of IPDs can only be achieved by identifying the underlying molecular pathology and thus, genetic testing might be introduced much earlier in the diagnostic process (►Fig. 1). This accounts particularly for patients with a bleeding tendency that is already indicative of a certain platelet dysfunction (e.g., GT), a positive family history, or conspicuous diagnostics in the functional testing mentioned before. Approximately half of all inherited thrombocytopenias, as well as some PFDs, are part of more complex syndromes, in which the platelet defect is accompanied by a high probability of clinically relevant alterations in other cell types, tissues, or organs (e.g., Wiskott–Aldrich syndrome, Hermansky–Pudlak syndrome [HPS]), or with a predisposition for developing a neoplastic disease (e.g., RUNX1- or ANKRD26-associated thrombocytopenia).[26–28] In patients in whom platelet function abnormalities are not isolated but syndromic, an accurate and complete diagnosis that reflects the underlying molecular pathology is important for future management.

From Candidate Screening to Whole Genome Sequencing

When identified variants are considered as relevant and disease-causing, it is important to perform segregation studies whenever possible. This strategy is especially useful

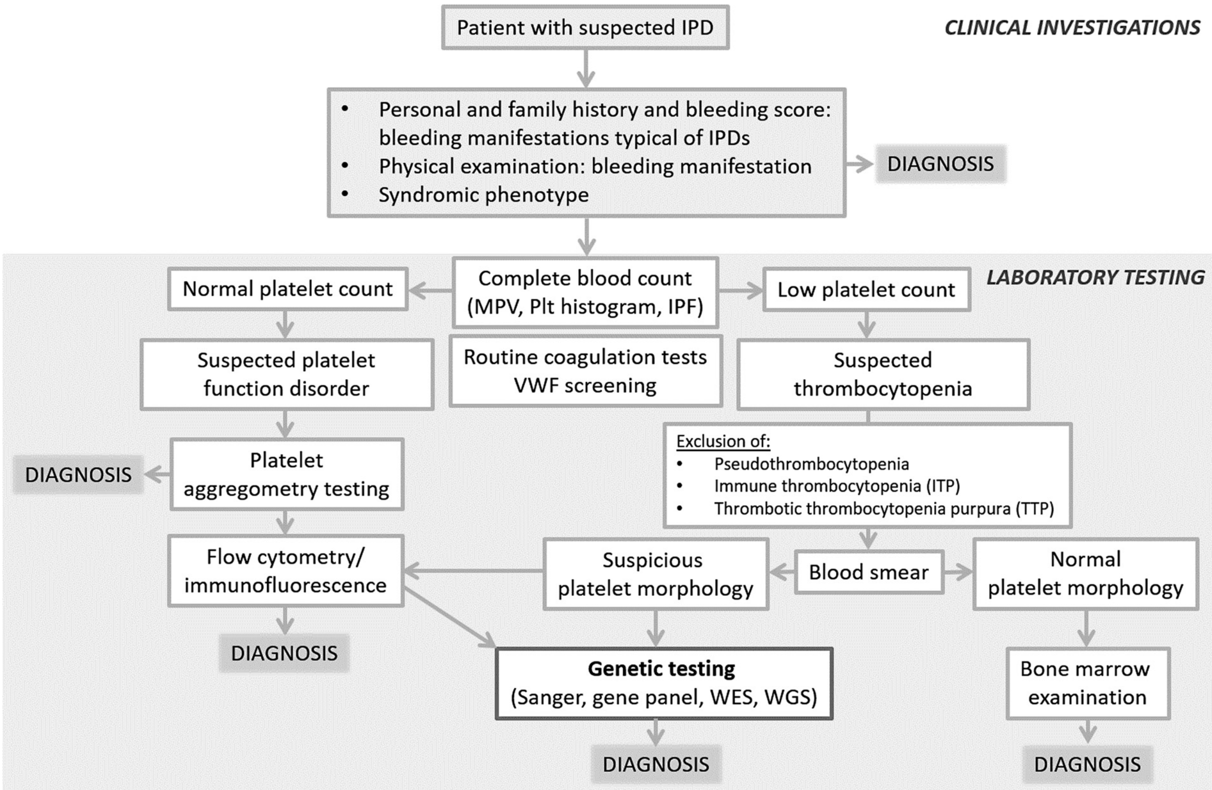

Fig. 1 Diagnostic algorithm for inherited platelet disorders (IPDs). IPF, immature platelet fraction; MPV, mean platelet volume; Plt, platelets; WES, whole exome sequencing; WGS, whole genome sequencing.

to confirm new variants and to improve the genotype–phenotype associations. It is, however, not applicable to cases with a nonspecific phenotype, where there is no obvious candidate gene, or when multiple genes are known to be causative (e.g., in cases of GT, BSS, HPS).[13,29] Although GT or BSS does not essentially require genetic testing for diagnosis, it will support to stratify genotype–phenotype associations and is indispensable for a (co-)segregation study in parents and siblings for potential genetic counseling to assist family planning. HPS is often diagnosed by the combination of laboratory findings (reduced platelet count, impaired platelet function, absence of platelet dense granules) and clinical features (oculocutaneous albinism, nystagmus).[30] Various genes have been identified to cause HPS, but dependent on the affected gene complex symptoms can be mild, whereas some mutations harbor additional risks like lung fibrosis.[30] The underlying gene as well as the gene variant may therefore be instrumental to determine when and how often future clinical screenings are recommended.

With the advent of HTS at the beginning of this millennium, a more accurate diagnosis of IPDs has become available.[31–33] Technical progress has made it possible to sequence preselected regions on the entire genome (targeted sequencing, panel sequencing), coding regions (whole exome sequencing) or the whole genome at rather low cost.[21] All sequencing approaches have several advantages and disadvantages, which have to be considered carefully before starting genetic testing (►**Table 1**). The HTS approach allows to receive information on large genes or groups of genes with a suspected disorder like GT, BSS, or HPS, without performing numerous Sanger sequencing reactions. There are typically groups of relevant genes analyzed depending on the underlying functional diagnostics that can readily identify variants in genes that are rarely analyzed. However, there are also some risks in HPS analysis: When large gene sets are analyzed at a time, the bioinformatics might result in many variants of unknown significance and associations could be made to quickly. The filter settings during the analysis will provide few or many putative genes that would all require a follow-up. For some genes, the coverage is still low; this needs to be specified and documented on the final report and, ideally, Sanger sequencing of these gene regions has to be performed to exclude that disease-causing variants remain undetected. Finally, it has to be specified which genes are analyzed: (1) unrelated oncogenic or preleukemic genes are not to be analyzed; (2) certain disease-relevant genes like *RUNX1*,[26] *ETV6*,[34] or *ANKRD26*[28] harbor an increased risk to develop leukemia. Patients (or parents) need to actively specify whether they want these genes to be looked at and whether they want to have this information revealed; (3) identified variants might not explain the underlying disease of the patient, but could have information on a carrier status that could become clinically relevant: heterozygous mutations in *F8* might not explain a platelet-based bleeding phenotype, but could become relevant when a (future) son inherited the affected gene and developed hemophilia. These options should be discussed before the analysis is performed to avoid confusion how to report these variants. When

Table 1 Advantages and disadvantages of different sequencing methods for genetic testing of IPDs

Sanger sequencing	High-throughput sequencing		
	Targeted gene panel sequencing	WES	WGS
Advantages			
Fast and cost-effective for low target number	Rapid and cost-effective for high target number (> 20 targets)	All genes analyzed in parallel	All genes analyzed in parallel
Established workflow	Analysis of disease-associated genes	Identification of new genes	Identification of new genes
Simple data analysis	Limited datasets		Identification of noncoding variants
Longer reads (500–700 bps)	Relatively inexpensive		Reliable detection of CNV
			Detection of structural variants
			Most uniform depth of sequencing
Disadvantages			
Low number of targets	New genes cannot be identified	Large datasets	Very large datasets
Not as cost-effective for high number of targets (>20 targets)	Noncoding variants are detected only if targeted	Limited detection of noncoding variants	Relatively expensive
Insensitive to CNV	Difficult detection of CNV	Difficult detection of CNV	Short reads (150–300 bps)
Low discovery power	No detection of structural variants	No detection of structural variants	Complicated analysis
	Requires updates as new disease-associated genes are discovered	Short reads (150–300 bps)	Risk of incidental findings
	Short reads (150–300 bps)	Relatively complicated analysis	
	Risk of incidental findings	Risk of incidental findings	

Abbreviations: bps, base pairs; CNV, copy number variation; WES, whole exome sequencing; WGS, whole genome sequencing.

patients agree to perform in research-based analyses, researchers, clinicians, and patients/parents should actively decide which information should be shared and which should not.

One obvious advantage of HTS is the small amount of blood needed and little risks of preanalytical artifacts. Buccal swabs are also possible for DNA isolation. Many research groups and consortia have already chosen HTS to accelerate the time to diagnosis or to identify novel genes and variants involved in the pathogenesis of IPDs.[13,32,35–38] Importantly, HTS has to meet specific quality criteria for diagnostic means (e.g., a minimum coverage per base; often 20–100 reads per base).[13,20] Inadequate coverage can be caused by GC-rich domains (often present in the 5′ region including the first exon), highly homologous regions, homopolymers, and repeats of any size.[39] Low-coverage areas should thus be filled in by orthogonal technologies, such as Sanger sequencing, to ensure adequate clinical sensitivity. Analytic achievements could be obtained by grouping so far known and functionally similar genes for suspected pathophysiology in one HTS gene panel. The THROMKIDplus Study Group, for example, developed, verified, and evaluated a targeted, panel-based next-generation sequencing approach comprising 59 genes associated with IPDs.[40]

Despite the progress in targeted HTS, a significant proportion of patients with IPDs still fail to receive a molecular diagnosis (i.e., detected pathogenic variant), despite being genotyped. In 2016, the THROMKIDplus panel-based sequencing approach provided a molecular diagnosis for only 26% of patients.[40] In the recently performed "Children with Inherited Platelet disorder Surveillance" study (CHIPS), in which 139 children with inherited thrombocytopenias were enrolled, the defective genetic locus could be identified in only 73 children (53%).[41] These low detection rates may reflect the fact that there is currently a lack of knowledge about all genes involved in the regulation of platelet production and function, and that other causative defects in noncoding genomic regions or in acquired gene defects for IPDs are overlooked by panel sequencing. One successful strategy for identifying new IPD disease genes, as well as for molecular diagnosis in established IPD genes, involves whole exome or whole genome sequencing, especially when combined with other selective approaches (e.g., with combined segregation analysis by Sanger sequencing) or complementary functional studies to ensure clinical relevance.[42–45] However, these analyses are still labor-intensive and require specialists with experience in bioinformatics analysis as well as suitable functional tests to interpret the relevance of novel variants.

Legal Basis and Ethical Aspects

The Swiss and German public health systems permit genetic analyses for diagnostic reasons since 2015 and 2016, respectively.[40] At that time, the German health care system covered the cost of a genetic diagnosis of up to 25 kilobases of coding sequence for patients with public health insurance, if a special letter of referral ("Überweisungsschein Muster 10") was provided, which typically allowed the analysis of groups of 5 to 10 genes. As of 2021, this restriction no longer applies, and analyses of larger panels or the entire exome to detect clinically relevant constitutional genomic mutations in the postnatal setting are covered by insurance ("EBM 11513—Postnatale Mutationssuche zum Nachweis oder Ausschluss einer krankheitsrelevanten oder krankheitsauslösenden konstitutionellen genomischen Mutation").

According to the Gendiagnostikgesetz (Genetic Diagnosis Act, GenDG), patients have to be properly informed about important ethical aspects, which arise with genetic testing (i.e., informed consent required). The ISTH has provided suggestions that can be modified to fulfill national guidelines and laws.[46] Variants in IPD-related genes like *RUNX1*, *ANKRD26*, or *ETV6* are associated with an increased risk for malignant disease (see the previous section). Handling of such unsolicited findings may bear an ethical dilemma for the attending clinician, since the patient's information self-determination is protected by the GenDG's basic principle: the right not to know. Although good genetic testing practice is ensured in this way, it bears the risk that pathogenic variants in IPD genes are not allowed to be analyzed and, thus, remain unidentified and finally not reported. These issues need to be carefully discussed with patients and parents, or with assistance of social (pediatric) specialists, often present in the respective clinical centers. Genetic testing may also reveal unexpected relationships between family members. Therefore, prior to a complex genetic analysis, patients have to be asked whether they want to receive unsolicited findings and they should be able to actively opt-in or opt-out.[46] Adequate patient education and documentation of informed consent according to the GenDG is thus fundamental and a legal requirement. In this context, children represent a particularly vulnerable group of patients, since they might be too young to participate in decision making.[47,48] In Germany, there are recommendations regarding predictive genetic testing in minors, that is, excluding testing of late-onset genetic disorders or carrier status (guideline by the *German Commission on Genetic Testing* [GEKO] section IV.3 [Bundesgesundheitsbl 2011, 54:1257–1261, DOI: 10.1007/s00103-011-1354-6] and guideline on genetics in minors by *German Society of Human Genetics* [https://doi.org/10.1007/s11825-007-0059-6]). According to the GenDG, genetic testing for differential diagnosis, in contrast, does not have any age restriction. Of note, there is often a misunderstanding regarding the clinical implications (where the GenDG is applicable) and (additional) biomedical research, which is explicitly exempted from the GenDG. Here, informed consent is also required, and studies are evaluated by a local or institutional ethical committee. Informed consent is typically age-grouped into minors of 8 to 12 years and 13 to 17 years, where the child's concerns and wishes are integrated. In long-lasting cohorts, a re-consent might be required once the child becomes 18 years old, especially when biomaterial (DNA) has been preserved in laboratories or biobanks.

Analysis

All identified variants need to be further evaluated and consolidated. It is pivotal to have "in silico filter settings" to a level that not too many variants are reported. All variants are stratified according to the recommendation provided by the *American College of Medical Genetics and Genomics* (ACMG).[49] In case an already reported variant associated with the same phenotype has been detected, the variant can readily be classified as "pathogenic" (class 5). Similar variants (same position, another amino acid change, or a neighboring position) might be considered as "likely pathogenic" (class 4). The biggest concern arises (1) when a variant (or a gene) has not yet been described, (2) where a gene has been rarely or not at all reported, (3) all evidence comes from animal studies, or (4) the overall evidence is limited. These variants of unknown clinical significance (VUS) (class 3) require further clarification, including a co-segregation analysis with parents, siblings, or other associated family members from the same pedigree or appropriate functional tests. Typically, class 3 variants should not be reported or only mentioned cautiously, to avoid that a genetic variant becomes associated with a person without being disease causative for the patient's phenotype. These variants usually remain in the patient file and do not only associate the patient with a nonpathogenic variant but also curb the interest to identify the correct genetic cause. Since genetic knowledge is expanding, a reanalysis or reinterpretation of variants is recommended after a few years.

Variants that are known to not cause the disease are grouped as "likely benign" (class 2) or "benign" (class 1). These variants are typically under-reported, as the effort to submit a variant to databases is less exciting when the result is negative (i.e., not explaining the phenotype). We highly encourage all clinicians and scientists to make the effort to report all new variants to databases.

The NCBI-hosted ClinVar public archive has installed expert panels to collect and continuously evaluate the evidence for genes and variants in multiple disease settings. The *Hemostasis/Thrombosis Gene Curation Panel* has been fully established in 2019 and since (March 2023) has curated 103 genes. Of note, "lumping and splitting considerations" are made to certain genes to account for the problem that for some genes like *MPL* a certain set of variants will cause one disease (CAMT; OMIM #604498), while other (mostly acquired) variants cause myeloproliferative disorders.[6] *GP1BA*, for example, is causative for two distinct disorders: BSS[9] and platelet-type von Willebrand disease.[50,51] In *ITGA2B*, the "classical" autosomal recessive variants lead to GT, while other variants act in an autosomal dominant way and cause the newly defined "platelet-type bleeding disorder 16" (OMIM #187800).[8] Genetic variants known to cause syndromic disorders might not be fully covered. The expert panel will consider both genetic and experimental (and animal-based) evidence according to a score sheet.[52] Finally, different classification is indicating whether the genetic evidence for the bleeding disorder is *definite*, *moderate*, or *limited*. The latter might occur when there are only few case reports in the literature and additional evidence from biological assays or mouse models is missing. Genes are continuously reevaluated, and the class of evidence might increase, but also decrease. For some genes like *P2RY12*, encoding for the platelet ADP receptor P2Y12, the entire evidence was surprisingly only *moderate*. The full list can be found at https://clinicalgenome.org/affiliation/40028/.

The most comprehensive list of genes is also compiled by the ISTH in the SSC *Subcommittee on Genomics in Thrombosis and Hemostasis*. Here, genes are classified according to gene curation properties and stratified into Tier 1, Tier 2, and Tier 3. Genes might move up or down between the tiers depending on the underlying evidence. The genes with the highest importance to study are the "GoldVariants."[52] This data can be found at https://www.isth.org/page/GinTh_GeneLists. New variants, especially from novel genes out of whole exome studies should be reported to this platform, so that evidence can accumulate, especially when results are not yet published.

Final Report

When identified variants are considered to be relevant and disease-causing, it is essential to perform segregation studies if possible (indispensable in case of class 3 [VUS] that cosegregates with other affected family members in the same pedigree, while unaffected members do not have this variant). The identified variants might also prompt additional functional tests that are typically performed in specialized laboratories. It is recommended to discuss complex patients in interdisciplinary case conferences. The final report should comprise information on the used technology and platforms. All tested genes need to be depicted with their RefSeq (NM) numbers of the analyzed transcripts. This allows to refer back to the exact sequence, in case new variant updates have been published in the meantime. Some exons still have a poor coverage in whole exome sequencing approaches, which needs to be declared in the final report. If possible (and available), an interpretation of the identified variant in respect to the anamnesis and functional diagnostics should be provided, otherwise the referral to a clinician or other experts is recommended. The patient might be informed in the setting of a human counseling ("humangenetische Beratung") according to §10 GenDG, but this is not mandatory.

Conclusion

IPDs, especially PFDs, are often complex disorders. Their diagnostics are overall poorly standardized and time consuming until a final diagnosis can be made and a genetic cause be attributed. Considering the high percentage of patients who are still left undiagnosed or poorly classified, there is an urgent need to significantly improve the rational approach for diagnosis of patients with suspected IPD. HTS allows to elucidate the cause of IPDs on the molecular level. Based on this, databases are continuously expanding and assisting to identify (non)-pathogenic variants. Future research on IPD genes will improve the diagnosis and treatment from which finally the IPD patients' quality of life will benefit.

Final Note

All Web links have been tested as active on May 18, 2023. The authors do not take responsibility for future changes of links or their contents.

Conflict of Interest

The authors declare that they have no conflict of interest.

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