Genome Medicine

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# Genome sequencing for the diagnosis of intellectual disability as a paradigm for rare diseases in the French healthcare setting: the prospective DEFIDIAG study

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## Abstract

**Background** Intellectual disability (ID) is the leading cause of patient referral to medical genetic departments in French academic hospitals. Whole genome sequencing (WGS) as a first diagnostic approach is expected to achieve a higher diagnostic yield than the French national reference strategies (RefStrategy) (fragile X expansion testing, chromosomal microarray analysis, and 44 ID genes panel), given its broad and more homogeneous coverage, its ability to identify copy number, structural and intergenic/deep intronic events.

**Methods** DEFIDIAG is a national, prospective pilot investigation, carried out in the framework of the French initiative for genomic medicine (*Plan France Médecine Génomique 2025*), aimed at comparing the diagnostic yield of WGS trio analysis (WGS-trio) (index case, father, mother) with the RefStrategy in real-life conditions of clinical and laboratory

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workflows. Both strategies were applied in a blinded fashion in 1239 ID probands (50% were already-tested, 50% were never-tested) with no definitive genetic diagnosis. Among them, a subgroup of 187 patients were randomized to undergo WGS-solo (proband only) in addition to WGS-trio and RefStrategy.

**Results** Four hundred forty two likely pathogenic/pathogenic single-nucleotide variants were identified (for 231 genes) as well as 171 variants of uncertain significance warranting clinical or functional reassessment for a potential reclassification (VUS+) (for 142 genes), 79 likely pathogenic/pathogenic copy number variants and 10 likely pathogenic/pathogenic structural variants. The diagnostic yield for likely pathogenic/pathogenic variants increased from 17.3% with the RefStrategy to 41.9% with WGS-trio in the never-tested patient cohort. An increase of 13.9% was observed in all categories by adding the VUS+, thus raising the yield to 56% for WGS-trio. Overall, WGS-solo enabled the identification of likely pathogenic/pathogenic variants in 29.9% of cases (increasing to 41.1% when including VUS+) compared to 21.9% with the RefStrategy. In addition, following recent reports of de novo variants in the non-coding spliceosomal *RNU4-2* gene as a common cause of ID, this gene was subsequently analyzed, leading to the identification of pathogenic de novo variants in 7 patients.

**Conclusions** As a first line test for ID diagnosis, WGS (including for solo situations) proved to be more effective than the reference strategy, in the context of real-life hospital settings in France.

**Trial registration** Prospectively registered with ClinicalTrials.gov under the identifier NCT04154891 (07/11/2019).

**Keywords** Whole genome sequencing (WGS), Short-read sequencing, Trio, Solo, Intellectual disability, Diagnostic yields, Real-life hospital setting, Centers of expertise, Multidisciplinary meetings

## Background

Rare diseases represent a global challenge for medical care, especially for identifying the underlying genetic causes, with sequencing technologies that have been evolving rapidly in recent years. The last decade has seen the advent of whole genome sequencing (WGS) as a rapidly growing and accessible diagnostic tool to be implemented in routine care. Worldwide efforts have been conducted to develop genomic research to improve the care of patients with rare diseases, prompting initiatives to deploy these technologies routinely, often at a national level. For instance, large-scale genomic research in the UK's 100,000 Genomes Project led by Genomics England [1–3] was followed by national implementation of WGS as a standard clinical service. Ensuring the best access to genomic medicine at a wider population level is embodied by the European Commission's "1+Million Genomes Initiative." Direct implementation in national settings has been considered, for example in Sweden [4], as well as in the Netherlands [5] and Germany [6].

In the same spirit, the launch of the French initiative for genomic medicine (*Plan France Médecine Génomique* 2025 PFMG 2025) [7] was designed to serve a wide range of medical disciplines (including cancer) for which pilot studies were designed. PFMG 2025 targets rare diseases as one of the key areas at the forefront of national WGS implementation, to improve patient care, shorten their diagnostic odyssey and boost research. The overarching goal of PFMG 2025 is to implement WGS in France by creating national clinical sequencing laboratories (two of which are now fully operational), and, in parallel, by conducting pilot studies (performed by research platforms, i.e., in this work, the national center for research in human genomics, *Centre National de Recherche en Génomique Humaine* (CEA-CNRGH)) to demonstrate the clinical utility of WGS in real-life healthcare pathways. Finally, the PFMG 2025 addresses key issues such as economic, societal, and ethical challenges, on a research basis. The pilot study, DEFIDIAG, focuses on intellectual disability (ID) as one of the most challenging, but also impactful, areas of rare diseases. ID affects around 1–3% of the general population, with around 15 per 1000 persons having mild ID and around 3 per 1000 having severe ID [8, 9]. It is the most common cause of referral to hospital-based clinical genetics centers. ID can appear as an isolated feature, thus non-syndromic. Alternatively, ID can be defined as syndromic if associated with: morphological developmental features (including facial gestalt) often associated with other developmental anomalies, additional neurodevelopmental features, such as epilepsy and autism spectrum disorder. ID is characterized by extensive genetic heterogeneity in more than 1700 genes [10] with numerous molecular pathogenic events reported in a wide range of categories, such as single-nucleotide variants (SNVs), small insertions or deletions (InDels), unbalanced (copy number variants, CNVs) or balanced structural variants (SVs) in both coding and non-coding regions, or even rarer events such as repeat expansions, uniparental disomies, and mobile element insertions [9]. Most ID patients with no molecular

diagnosis in France still undergo a first basic investigation (fragile X expansion detection and chromosomal microarray analysis (CMA)) with a diagnostic yield of less than 20% [11]. This first line testing (at the time of our study design) is usually followed by additional analyses using an ID gene panel, containing a core set of 44 genes, namely the 44GPS minimal list recommended by the French national association of molecular genetics practitioners (*Association Nationale des Praticiens de Génétique Moléculaire, ANPGM*) (see Additional file 1). This provides an additional diagnostic yield of 10–12% [12, 13], rising to 40% with whole exome sequencing (WES), as in other studies [14–18]. Indeed, in several countries, WES as the first line test for the diagnosis of rare genetic diseases has been shown to be more time- and cost-effective than panels for children with suspected monogenic disorders [19–24]. There is growing evidence of the effectiveness of WGS compared to standard panel testing and WES. WGS covering coding and noncoding sequences enables detection of a much wider range of molecular events, such as: (1) SNVs and InDels in coding regions (even in GC rich regions), (2) 5′ and 3′ UTRs (untranslated regions), promoters or deep intronic regions [25–30]; (3) unbalanced chromosomal anomalies (CNVs), with greater accuracy due to homogeneous coverage; (4) balanced SVs, such as inversions and translocations; and lastly, (5) mechanisms still observed infrequently, such as uniparental disomy for imprinted chromosomal regions, repeat expansions or mobile element insertions [5, 31–35].

A recent worldwide meta-analysis carried out over the past decade, which has seen constant technological improvement in WGS, notably economic and practical accessibility, showed that WGS for rare diseases had a higher diagnostic yield than WES, and predicted wider use of WGS in clinical settings [36]. A retrospective benchmarking study of 1000 patients previously diagnosed with rare disease confirmed the usefulness of WGS as first-line strategy in genetic diagnostic laboratories [37]. WGS is now widely recognized as a superior diagnostic approach compared to traditional methods. It has been recommended that WGS replace chromosomal microarray analysis (CMA) and *FMR1* testing as the first-line genetic test in individuals with ID or neurodevelopmental disorders (NDD), with a diagnostic yield of up to 35% when used as an initial investigation [38]. Furthermore, WGS has demonstrated significant clinical utility in pediatric patients with previously unexplained ID, even after prior testing with WES and CMA. In this context, the diagnostic yield ranges from 21 to 26% [38, 39]. In line with these findings, the American College of Medical Genetics and Genomics (ACMG) now recommends the use of genomic sequencing as a first- or second-line diagnostic test for individuals with congenital anomalies and/or ID [40].

However, the value of WGS as the first all-in-one test proposed in ID has yet to be confirmed at a national level, specifically in the French healthcare setting (in the context of the National Plan for Rare Diseases, *Plan National Maladies Rares*—PNMR). Indeed, marked differences exist between countries in terms of the technologies used and in the organization of clinical, biological and bioinformatics pathways [41]. In addition, the heterogeneity in the causes and presentation of ID in different populations, and the difficulty of transferring results of medical-economic studies from one country to another due to differences in the organization of healthcare systems, methods of financing care, and variability in the costs incurred by patients and their families, make it challenging to automatically generalize results concerning WGS utility and efficiency to all national settings [42].

As a use case for genomic medicine in the French healthcare system, the DEFIDIAG research program aims to assess broadly the added value of WGS in (1) improving the diagnostic yield, compared to the French Reference Strategy (RefStrategy), which is the primary focus of this paper; (2) demonstrating its efficiency in terms of cost per additional positive diagnosis (also currently being addressed in an ongoing dedicated health economic study), and (3) showing the impact in terms of care modification for ID patients and on the life experience for parents and caregivers [43, 44].

DEFIDIAG is a prospective multicenter diagnostic clinical research study, based on 1239 patients with ID and their biological parents (in total, 3717 subjects included and with WGS performed). The primary objective was to compare the percentage of causal genetic diagnoses identified by WGS performed on a trio (patient and both parents, WGS-trio), to that obtained using the current French reference minimal recognized strategy (fragile X expansion detection, CMA and 44GPS—RefStrategy). Two populations were studied in a real-life routine diagnostic workup: never-explored ID patients (NeverTested) attending a first genetic consultation and already-explored patients (AlreadyTested) attending a follow-up consultation (Fig. 1 and Additional file 2: Fig. S1). The main secondary objectives were to evaluate the diagnostic yield of WGS in solo situations (WGS-solo) compared to trios (WGS-trio); to assess WGS-trio diagnostic yield in various clinical subgroups of ID patients, and to assess detection of causal structural changes (such as CNVs or SVs). The strengths of this study, based on 2 patient cohorts (NeverTested and AlreadyTested), lie in part in its methodology, which notably included (1) comparison of 3 strategies (WGS-trio, WGS-solo, RefStrategy), (2) blinded interpretation of the 3 strategies, to limit

verification bias due to a national uniform organization, (3) two independent WGS interpretations (performed by 2 independent laboratories), followed by a global multidisciplinary meeting (MDM) discussion in which the RefStrategy results were discussed first, then the WGS-solo (when applicable), and finally the WGS-trio results, leading to a conclusion for each patient; (4) evaluation of the NeverTested population to extrapolate for future first-line diagnostic WGS use; (5) identification of workflow conditions close to routine care and in a large number of patients in the perspective of future broad national WGS implementation.

## Methods

### Description of DEFIDIAG investigating sites and professionals involved

In France, since 2004, rare diseases have been recognized as a public health priority with successive National Plans for Rare Diseases (*Plan National Maladies Rares*, PNMRs) [45] implemented to improve patient care pathways by creating, among others, clinical and laboratory expert centers, so-called "reference centers" (*Centres de Référence Maladies Rares*) as well as national networks (*Filières de Santé Maladies Rares*). As ID is the most common cause of referral to pediatric medical genetics departments, ID care is embedded in dedicated rare disease reference centers that are aggregated into two national networks: AnDDI-Rares [46] and DéfiScience [47].

In this context, DEFIDIAG recruitment centers comprised the medical genetics departments of 14 university hospitals (UH), ensuring nationwide geographic coverage (i.e., UH Angers, UH Bordeaux, UH Dijon, UH Grenoble, UH Lille, UH Lyon, UH Montpellier, UH Nantes, UH Paris [Necker Hospital & *Imagine* Institute, La Pitié-Salpêtrière and Armand-Trousseau Hospitals], UH Marseille, UH Rennes, UH Rouen and UH Strasbourg). These departments were in charge of patient recruitment and

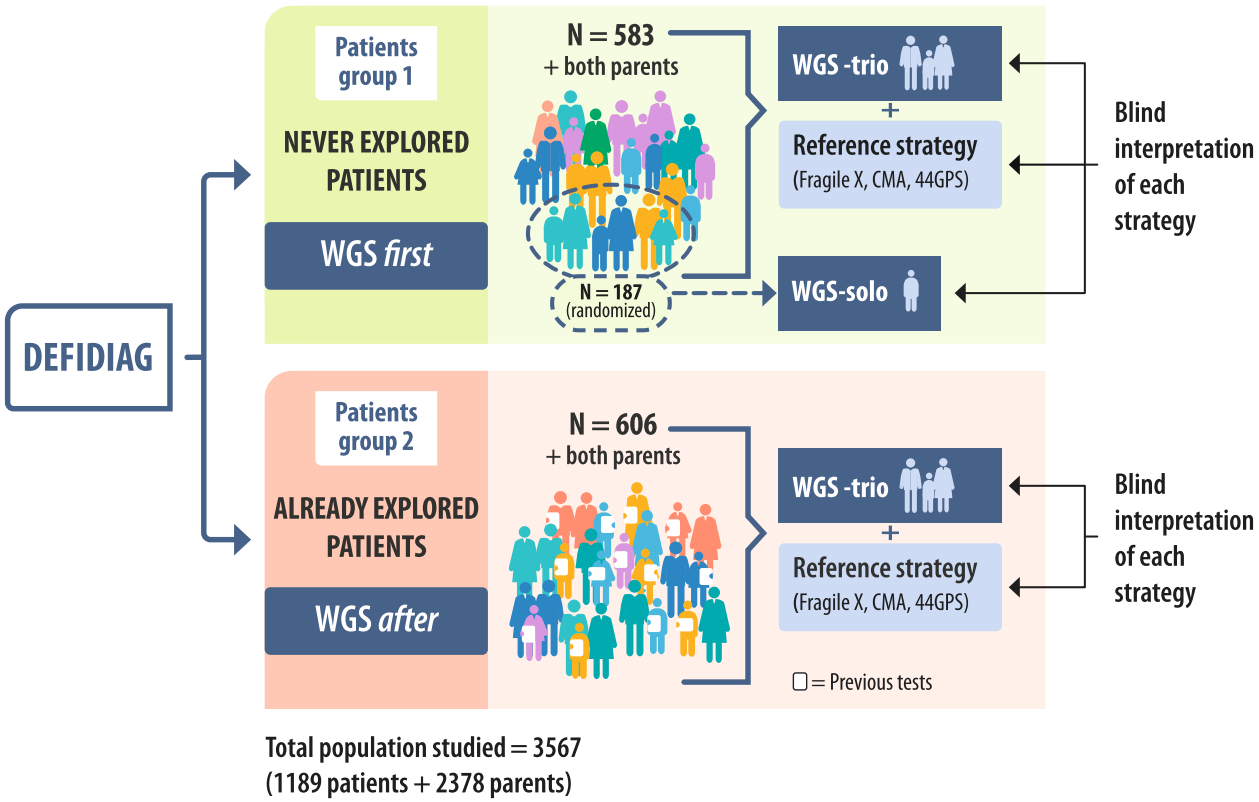


**Fig. 1** Overview of the DEFIDIAG study. A schematic overview of the DEFIDIAG study is presented. The starting point is patients with ID and their parents (the trio), referred to the medical genetics department of their local university hospital. Two distinct patient groups were defined, both of which underwent WGS in trio (WGS-trio) in addition to the reference strategy (Fragile X analysis, CMA, and the 44GPS genes panel). Patient group 1 included patients who had never undergone any genetic analysis ($n = 583$), for whom WGS-trio was performed as the first-line investigation (WGS *first*). Among them, a randomized subgroup of 187 patients also underwent WGS in solo (WGS-solo). Patient group 2 included patients who had already undergone previous genetic investigations, but without identification of the cause of their ID ($n = 606$), for whom WGS-trio was performed as the second- or third-line investigation (WGS *after*). Each patient included underwent both WGS and the reference strategy, with blinded interpretation. 44GPS: 44-ID genes panel; CMA: chromosomal microarray analysis; WGS: whole genome sequencing

inclusion following their usual workflow, and both NeverTested and AlreadyTested (limited to 50% of inclusions) patients were included through standard consultations. The referring clinicians were the ultimate recipients of the molecular research report to be delivered back to the patients and their families, by way of a standard restitution consultation to announce the results.

The study also involved 6 UH expert genetic diagnostic laboratories for the reception of patient blood samples, DNA extraction and transfer to CNRGH, reception of bioinformatics data, interpretation and variant validation, analysis of trios versus solos (alternating between them as lead or mirror laboratories to ensure double blind analysis), driving a multidisciplinary meeting with the clinicians, and delivering a final molecular research report to the referring clinician as per usual UH practice.

Overall, the study involved around 200 health professionals across the country (mostly clinical geneticists, laboratory geneticists, genetic counselors, neuropsychologists, neuropaediatricians), all are members of the DEFIDAG study group. In addition, genomic scientists (bioinformatics specialists, molecular biologists) were involved via the 6 expert laboratories, the sequencing center (CEA-CNRGH) and a bioinformatics laboratory for the Polyweb interface (provided by the *Imagine* Institute).

The study was prospectively registered with ClinicalTrials.gov under the identifier NCT04154891 (07/11/2019).

#### Methodology of the study

The methodology of this diagnostic clinical study has previously been described [43] (the study timeline is shown in Additional file 2: Fig. S2). Briefly, for each inclusion (patient and parents) done by a clinical center of expertise, a unique set of genomic data sequences was produced by a single sequencing platform (CNRGH, Evry, France). Genomic data analyses were then performed blindly by two independent mirror hospital laboratories: the first laboratory analyzed the WGS-trio, while the second laboratory analyzed only the 44GPS (panel extracted in silico from the WGS), as well as WGS-solo for a randomized selection of patients. Fragile X expansion detection and CMA (included in the RefStrategy) was performed by the routine care circuit, mainly independent from the WGS DEFIDIAG pathway [43]. Following the multidisciplinary meeting a report is provided back to the referral medical center.

#### Patient recruitment and inclusion

ID patients consulting a clinical geneticist in one of the participating centers were systematically screened for eligibility (see detailed inclusion and exclusion criteria in Additional file 3: Table S1) and invited to participate in the DEFIDIAG study. The study included children and adults diagnosed with ID of unknown etiology (patients with highly likely clinical well-known diagnoses of ID syndromes were not included, such as Williams-Beuren syndrome or Down syndrome for instance, as these cases would have undergone a direct targeted diagnostic test). Patients with all levels of ID, if possible proven by validated neuropsychological standard testing (Wechsler Preschool and Primary Scale of Intelligence (WPPSI IV), Wechsler Intelligence Scale for Children (WISC V), Wechsler Adult Intelligence Scale (WAIS IV) or Vineland Adaptive Behavior Scale – II (VABS-II)) were considered for inclusion, whether or not they had other associated features (including autism spectrum disorder, brain malformations, epilepsy). Because ID testing can be challenging for children aged between 0 and 5 years, only cases with severely delayed development in terms of motor skills, language, and/or sociability were included for this age range. The study included stratification according to patient age (< 2 years old, 2–5 years old, and > 5 years old), severity of ID, and associated features (CNS developmental anomalies, epilepsy, or other non-CNS anomalies).

#### Objectives, main endpoints, and sample size calculation

The primary objective was to compare the percentage of causal genetic diagnoses identified by WGS-trio with that achieved using the current RefStrategy in ID patients attending a first genetic consultation (NeverTested). The main endpoint was the identification of a causal diagnosis of ID defined as the identification of one or more likely pathogenic or pathogenic variant(s) that explained the symptoms presented by the patient (following standard biological interpretation criteria, including ACMG recommendations [48–51]) and validated during a dedicated DEFIDIAG MDM. The main secondary objectives were to evaluate the diagnostic yield of WGS in solo situations (WGS-solo) compared to trios (WGS-trio); to assess WGS-trio diagnostic yield in various clinical subgroups of ID patients, and to assess detection of causal structural changes (such as CNVs or SVs); incremental cost-effectiveness ratio, expressed in terms of cost per additional positive diagnosis; estimated mean cost of diagnostic wavering; percentage with at least one modification in medical, medico-social, rehabilitative and psychological follow-up; and median time to obtain results.

The sample size was calculated as previously described [43]. Briefly, it was calculated to provide 80% power for the comparison between WGS-trio and RefStrategy in the NeverTested group. It also aimed to ensure sufficient power in subgroups defined by age (three groups: < 2 years, 2–5 years, > 5 years), severity of intellectual disability (ID), and associated manifestations (four

groups, namely: (1) mild ID with another sign, (2) moderate to severe ID, (3) ID with major noncerebral abnormality, and (4) ID associated with epilepsy).

In addition, comparison of the diagnostic yield of WGS-trio and WGS-solo in a subgroup of randomized NeverTested patients was planned. In order to counteract inflation of the alpha risk due to multiple testing, Bonferroni correction was applied, and the alpha risk was set at 0.0056 (bilateral formulation) to account for the 9 main comparisons planned. The sample size based on this alpha level was determined based on an expected difference of 15% in the smallest subgroup, expected to represent 15% of the NeverTested population (subjects with mild ID associated with another significant sign), a hypothesized difference of 15% between the WGS-trio and the RefStrategy in this particular subgroup and a fewer than 1% of diagnoses identified with the RefStrategy but not identified by the WGS-trio. Under these hypotheses, we estimated that it was necessary to screen 607 NeverTested patients. We planned to include the same number of AlreadyTested and it was expected that 5% of samples would not be analyzable, and thus a total of 1275 index cases as well as both their parents had to be included (i.e., a total of 3825 participants). A subgroup of 196 patients was randomized to undergo WGS-solo in addition to WGS-trio plus RefStrategy (including 187 patients with a result for all 3 strategies). Additional file 2: Fig. S1 presents the overall flowchart of the study.

#### Study data collection, storage, and analysis

Clinical data and the results of the genetic analyses (including analyses prior to the study that were not communicated to the DEFIDIAG laboratories) were recorded in a dedicated electronic Case Report Form (e-CRF) designed by the CIC1432 (Dijon-Bourgogne University Hospital & Inserm Clinical Investigation Center—Clinical Epidemiology Unit) using CleanWEB software [52]. The use of CleanWEB software enabled detection of missing and incoherent information through automated data checks, which generated queries immediately after data entry. Requests for corrections were also generated by CIC 1432 team and sent to the recruiting center and/or the reference laboratory until data were as complete, consistent and in line with the source data as possible.

#### WGS sequencing

DNA was extracted from whole blood samples by one of the 6 reference DEFIDIAG University Hospital diagnostic laboratories (each clinical department being linked to one of the 6 hospital laboratories), and 3 µg was sent to the national research sequencing center (CEA-CNRGH) for centralized WGS. Samples were sent in barcoded tubes, enabling accurate sample tracking during the whole process (unique barcodes, scanned at each stage of sample use). A full quality control was performed on each DNA sample (fluorimetric DNA quantification, in duplicate; DNA integrity evaluation using the DNA integrity number (DIN); PCR amplification test and gender control). All samples with at least 1 µg available after quality control and a DIN value greater than 6 were used to prepare libraries for short-read WGS using the Illumina TruSeq DNA PCRFree kit (Illumina Inc., San Diego, CA, USA) on the CEA-CNRGH automated platform. Sequencing was then performed as paired-end 150-bp reads on an Illumina NovaSeq6000, with libraries pooled in order to reach a mean coverage of 30 × for each sample.

#### WGS analysis, data generation and transfer

WGS sequences were analyzed by two separate SV and SNV/indel pipelines developed and validated by the CEA-CNRGH team (see Additional file 4) and the *Imagine* Institute bioinformatics team (Polyweb interface [53]). Briefly, raw data were produced as compressed FASTQ files generated from the.bcl files by the CEA-CNRGH. The sequences were aligned to the human reference genome GRCh37 using the Burrows-Wheeler Aligner BWA software [54] (version 0.7.17) and made available as BAM files. Aligned sequences were sorted and cleaned, and the PCR duplicates were marked using Sambamba software [55] (version 0.8.1) in order to eliminate well-known biases inherent to NGS. Local realignment of the sequences around insertion and deletion sites and base quality recalibration were performed using GATK software [56] (version 4.1.8.0). After sequence quality control and alignment to the reference genome, the CEA-CNRGH performed the variant calling on the entire genome for the SNVs, indels, and SVs (including CNVs). SNV and indel calling was performed using the Haplotype Caller from GATK software (version 4.1.8.0) in "bp resolution" mode to produce gVCF files. CNV detection > 1 kb was performed using three different software packages: WisecondorX [57] (version 1.2.4), Canvas [58] (version 1.40.0.1613), and Manta [59] (version 1.6.0). Balanced SV (translocations, inversions) detection was done using Manta software (version 1.6.0). Results were produced in VCF format to match the common file standard format in NGS analysis. These files were then collected by the *Imagine* Institute Polyweb platform: additional combined trio gVCF analysis (genotypeGvcf) and CNV WisecondorX analysis were performed.

The full study protocol is provided in Additional File 5.

### Variant annotation

The .VCF and .BAM files were imported into the Polyweb software that was previously developed and validated by the *Imagine* Institute bioinformatics platform [53]. Polyweb enables the annotation, analysis, and visualization of all the genomic variations in two different web interfaces: PolyViewer (for SNVs, small indels, exonic deletions or duplications) and PolyCyto (for balanced and unbalanced SV) of all human genes in trio or solo analysis. Moreover, the use of an in silico bioinformatics filter made it possible to study only variations from the 44GPS gene panel. The sequence reads were visualized using the IGV software [60]. Uniparental disomies and short tandem repeat (STR) expansions were not investigated.

#### *SNV/indel analysis*

Variants were annotated using the Paris-Cité University-IMAGINE institute in-house annotation pipeline, which is integrated into PolyWeb, the proprietary variant analysis, and visualization platform. This pipeline combines data from commonly used free-access databases (GnomAD [61], Clinvar [62], OMIM [63], GENCODE [64]), the licensed HGMDpro database [65], and an internal resource, namely *Déjà Vu*. The in-house annotation pipeline also incorporates gene and protein impact predictions, splice prediction (SpliceAI [66]), pathogenicity scores (CADD v1.6 [67], PolyPhen-2 [68], SIFT [69], REVEL [70]), and, in the context of trio analysis, assesses inheritance patterns and sequencing data (number of mutated and total reads). The internal database (*Déjà Vu*) contains over 20,000 exomes, 50,000 panels, and 1000 genomes for SNVs/indel variations with differentiation between ID and non-ID patients.

The following default filtration keys were applied to focus on potentially pathogenic variations: GnomAD allele count < 1000, GnomAD homozygote count < 10, and predicted protein impact onto all gene transcripts (stop gain, stop loss, start loss, frameshift, in frame deletions or insertions, missense, and predicted splice region, *Déjà Vu* for non-ID patients < 50 and homozygote count < 10).

Ranking of identified variations was then performed based on internal Polyweb criteria: variation sequence quality de novo status if available, known ID gene or OMIM gene, protein or splicing impact prediction, genes with autosomal recessive inheritance and homozygous or compound heterozygous variations, males and X-linked variations, known pathogenic variations in HGMDpro or ClinVar, frequency in GnomAD. Those criteria ensured that all variations were analyzed from all known human genes (OMIM referenced or not) that are predicted to affect proteins.

#### *SV analysis*

The PolyCyto interface gives access to several annotations using AnnotSV software [71], DGV (database of genomic variants) [72], OMIM, and internal *Déjà Vu* databases. At the time the project started, the internal SV database (*Déjà Vu*) contained 200 Novaseq 6000 sequenced genomes from non-ID patients and has now increased up to 5000 (mostly ID patients).

Ranking of identified variations is based on calling quality and inheritance status. For balanced SVs (translocations and inversions), a greater weight is given to variations whose break-points are found in OMIM genes.

After filtering CNVs already detected at least 20 times in the *Déjà Vu* database, all detected CNVs were analyzed using standard criteria [ACMG recommendations [49]]. For *Déjà Vu* count, two CNVs were considered identical if they overlapped over 90% of their reciprocal length. For balanced SVs (translocation and inversion), break-points must have an identical genomic position ± 50 bp.

All imbalanced and balanced SV were checked in IGV software by visualizing paired read alignment anomalies (insert size, pair orientation) and split reads. In addition, for CNVs, allele frequency plots ranked according to chromosomal positions were also available (SNP array like visualization).

#### *RNU4-2 analysis*

Following recent reports about de novo variants in the non-coding spliceosomal snRNA gene *RNU4-2* as a common cause of ID, this gene was secondarily explored (after all other data had been closed) specifically on the Polyweb interface [73–76].

### Clinical variant interpretation

Each multidisciplinary meeting included clinical geneticists from the recruiting centers, other clinicians in charge of the patients' follow-up, genetic counselors, molecular and chromosomal geneticists (from both the lead reference laboratory and its mirror laboratory). To ensure a reasonable number of cases to be reviewed by each multidisciplinary meeting, three independent meetings were organized in parallel, each of them grouping two laboratories and four clinical centers. Each multidisciplinary meeting reviewed in total about 400 inclusions. Each meeting was organized according to the following format: discussion of the list of variants of interest obtained by the simplex 44GPS analysis; then by WGS-solo analysis (for the 187 randomized patients); and finally, by WGS-trio. At each step, any additional confirmation analysis that could be required in the course of standard care (Sanger, qPCR, FISH, mRNA analysis) was recorded on the multidisciplinary meeting report and

in the e-CRF, for further subsequent medico-economic evaluation. A conclusion concerning the pathogenicity of variant(s) identified by the different approaches was reached during the session [43].

#### *Confirmation of detected genomic aberrations by a secondary method*

If additional confirmation methods were required, the reference laboratory was in charge of this analysis and the case was subsequently reviewed at the next multidisciplinary meeting.

#### *Reporting of results*

The final results (likely pathogenic and pathogenic variants) were recorded in a molecular research report sent to the clinical geneticist in charge of the case. Variants occurring in new genes and putative variations with no clear-cut pathogenic effect in known genes requiring further functional validation were classified as VUS + until the end of the project. Potential reclassification was managed using standard care procedures (including literature review and replication cohorts by way of international collaborations but also splice effect analyses, epigenetic signature testing and other ad hoc functional studies).

#### *Biological function study of genes involved in the DEFIDIAG cohort*

To calculate the functional enrichment of DEFIDIAG-ID genes ($n = 231$), the method described by Kochinke et al. was reproduced and the SysNDD gene database was used as a background set. The latest known ID-associated genes were retrieved by querying the SysNDD database (version 2024/08) [77] using Phenotype = "Intellectual disability" and Category = "Definitive" as parameters and a list of 1685 SysNDD-ID genes was obtained. Next, the gene ontology (GO) terms of DEFIDIAG and SysNDD gene sets were retrieved using the UniProt API portal and the annotated genes were then distributed into the 32 SysNDD-defined functional categories [77], according to their associated GO terms. A Fold Enrichment of DEFIDIAG-ID-genes against the SysNDD genes was calculated for each functional category, and the statistical significance was calculated using Fisher's exact test applying the Benjamini–Hochberg method.

#### *Quality assurance, development, and innovation*

Several quality controls were carried out before biological analysis: genome mean coverage over 25× was required for the trio; sex verification (*SRY* detection) and trio concordance (< 1% of Mendelian error transmission in trio using PLINK software [78]) were checked before interpretation.

## Results

### Description of the study population

Overall, among 1786 patients screened, 1275 were considered eligible, and 1239 were finally included in the DEFIDIAG study between March 2020 (first inclusion) and April 2022 (last inclusion), including a period of interruption linked to the Covid-19 lockdown (from March to June 2020) (Fig. 1 and Additional file 2: Fig. S2).

The characteristics of both groups (NeverTested and AlreadyTested) are presented in Table 1.

### Genetic testing

Results for the RefStrategy and WGS-trio were obtained for 583 NeverTested patients (including 187 patients randomized for WGS-solo) and 606 AlreadyTested patients (Fig. 1 and Additional file 2: Fig. S1). Among the AlreadyTested patients, 92.7% had previously undergone CMA, 38.3% an ID gene panel, and 15.2% a WES (some patients had undergone 2 or more of these investigations).

### Demographic characteristics

AlreadyTested patients were older than NeverTested patients ($p < 10^{-4}$) and the male/female ratio was 1.82 in NeverTested vs 1.31 in AlreadyTested ($p = 0.006$).

### ID heterogeneity

The first parental concerns regarding signs suggestive of ID or developmental delay (early hypotonia, motor delay, speech delay) were recorded before 3 years old in most patients: 82.5% in the NeverTested group and 91.2% in the AlreadyTested group. Neuropsychological testing was performed in 63% of index cases and showed that ID severity was milder in the NeverTested group compared to the AlreadyTested group ($p < 10^{-4}$). This is consistent with the fact that brain malformations, hypotonia, and epilepsy were more frequently reported in AlreadyTested patients (20.8%, 36.3%, and 29.4%, respectively) (Table 1).

The clinical DEFIDIAG data reflect the well-known heterogeneity of the ID patient population, including 22.5% with isolated ID, compared to 77.5% with syndromic ID (74.9% NeverTested; 80% AlreadyTested, $p = 0.036$), which included dysmorphic features, other developmental anomalies, hearing and ophthalmological anomalies (Table 1).

### Diagnostic yield

#### *Diagnostic yield in the NeverTested vs AlreadyTested groups*

Regardless of the clinical subgroup (according to age, ID severity or associated manifestations), diagnostic yields with WGS-trio were significantly higher than with the RefStrategy in both the AlreadyTested and NeverTested groups (see Additional file 3: Table S2).

**Table 1** Description of the study population (DEFIDIAG study, 2020–2023)

| Index case characteristics | NeverTested patients (*n* = 583) | | AlreadyTested patients (*n* = 606) | | Randomized patients (*n* = 187 NeverTested) | |
|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | *n* | % |
| ***Age at inclusion*** | | | | | | |
| < 2 years | 52 | 8.9 | 27 | 4.5 | 20 | 10.7 |
| [2–5] years | 240 | 41.2 | 158 | 26.1 | 78 | 41.7 |
| [6–18] years | 192 | 32.9 | 304 | 50.2 | 61 | 32.6 |
| ≥ 18 years | 99 | 17.0 | 117 | 19.3 | 28 | 15.0 |
| ***Sex*** | | | | | | |
| Male | 376 | 64.5 | 344 | 56.8 | 116 | 62.0 |
| Female | 207 | 35.5 | 262 | 43.2 | 71 | 38.0 |
| ***ID severity (4 MD)*** | 579 | 99.3 | 606 | 100.0 | 187 | |
| Profound ID | 15 | 2.6 | 47 | 7.8 | 5 | 2.7 |
| Severe ID | 134 | 23.1 | 199 | 32.8 | 38 | 20.3 |
| Moderate ID | 187 | 32.3 | 187 | 30.9 | 66 | 35.3 |
| Mild ID | 243 | 42.0 | 173 | 28.5 | 78 | 41.7 |
| ***Age at first signs (4 MD)*** | | | | | | |
| < 3 years | 481 | 82.8 | 553 | 91.6 | 153 | 81.8 |
| [3–12] years | 98 | 16.9 | 51 | 8.4 | 33 | 17.6 |
| [12–18] years | 2 | 0.3 | 0 | 0.0 | 1 | 0.5 |
| **Family history** | | | | | | |
| Consanguinity | 45 | 7.7 | 33 | 5.4 | 16 | 8.6 |
| Other cases of ID in the family | 166 | 28.5 | 148 | 24.4 | 57 | 30.5 |
| **Pregnancy** | | | | | | |
| Medically assisted reproduction | 16 | 64.0 | 21 | 61.8 | 6 | 66.7 |
| Maternal events during pregnancy (6 MD) | 85 | 14.7 | 67 | 11.1 | 28 | 15.1 |
| Maternal diabetes | 57 | 67.1 | 38 | 56.7 | 17 | 60.7 |
| Alcohol consumption | 2 | 2.4 | 1 | 1.5 | 0 | 0.0 |
| Medication intake | 22 | 25.9 | 22 | 32.8 | 8 | 28.6 |
| Addiction to drugs | 7 | 8.2 | 8 | 11.9 | 4 | 14.3 |
| **Prenatal history** | | | | | | |
| Abnormal prenatal development (4 MD) | 80 | 13.8 | 131 | 21.7 | 26 | 14.0 |
| Intrauterine growth restriction (4 MD) | 32 | 5.5 | 57 | 9.4 | 7 | 3.8 |
| **Characteristics at birth** | | | | | | |
| ***Gestational age (46 MD)*** | 562 | | 581 | | 178 | |
| < 32 WG | 12 | 2.1 | 17 | 2.9 | 3 | 1.7 |
| 33–37 WG | 84 | 14.9 | 92 | 15.8 | 24 | 13.5 |
| > 37 WG | 466 | 82.9 | 472 | 81.2 | 151 | 84.8 |
| ***Complications (12 MD)*** | 155 | 26.8 | 177 | 29.5 | 41 | 22.0 |
| ***APGAR < 10 at 5 min (169 MD)*** | 56 | 11.2 | 71 | 13.7 | 17 | 10.6 |
| **Manifestations associated with ID** | | | | | | |
| ***Developmental anomalies (facial dysmorphism/malformation – 4 MD)*** | 435 | 74.9 | 483 | 80.0 | 136 | 72.7 |
| ***Major non cerebral abnormality (24 MD)*** | 71 | 16.6 | 92 | 19.7 | 18 | 13.3 |
| ***Cerebral malformation (37 MD)*** | 48 | 11.6 | 97 | 20.8 | 14 | 10.7 |
| ***Epilepsy*** | 89 | 15.3 | 178 | 29.4 | 29 | 15.5 |
| Age at diagnosis (years; median (Interquartile range)) | 2.0 | (0.9–7.0) | 1.5 | (0.6–5.0) | 1.5 | (0.9–4.0) |
| Treated (1 MD) | 78 | 87.6 | 157 | 94.0 | 25 | 86.2 |
| Drug-resistant (11 MD) | 16 | 21.6 | 42 | 28.0 | 6 | 24.0 |
| ***Other signs*** | 438 | 75.1 | 465 | 76.7 | 147 | 78.6 |

**Table 1** (continued)

| Index case characteristics | NeverTested patients (*n* = 583) | | AlreadyTested patients (*n* = 606) | | Randomized patients (*n* = 187 NeverTested) | |
|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | *n* | % |
| Autism spectrum disorder | 173 | 39.5 | 170 | 36.6 | 60 | 40.8 |
| Behavioral disorders | 102 | 23.3 | 123 | 26.5 | 37 | 25.2 |
| Anxiety disorder | 101 | 23.1 | 138 | 29.7 | 35 | 23.8 |
| Hyperkinesia | 101 | 23.1 | 106 | 22.8 | 37 | 25.2 |
| Sleep disorders | 126 | 28.8 | 139 | 29.9 | 46 | 31.3 |
| Eating disorders | 118 | 26.9 | 108 | 23.2 | 36 | 24.5 |
| Hypotonia | 125 | 28.5 | 169 | 36.3 | 38 | 25.9 |
| Pyramidal syndrome | 27 | 6.2 | 36 | 7.7 | 7 | 4.8 |
| Ataxia | 22 | 5.0 | 35 | 7.5 | 9 | 6.1 |
| Abnormal movements | 51 | 11.6 | 47 | 10.1 | 15 | 10.2 |
| Oculomotor disorders | 35 | 8.0 | 44 | 9.5 | 11 | 7.5 |
| Neuropathy | 4 | 0.9 | 9 | 1.9 | 3 | 2.0 |
| Myopathy | 1 | 0.2 | 7 | 1.5 | 0 | 0.0 |

*ID* Intellectual disability, *MD* Missing data, min, minutes, *WG* Weeks of gestation

Overall, the diagnostic yield for likely pathogenic/pathogenic variants was as follows:

- For the RefStrategy: 17.3% in NeverTested patients and 6.3% in AlreadyTested patients (Fig. 2A).
- For the WGS-trio: 41.9% for the NeverTested patients and 42.2% for the AlreadyTested patients (Fig. 2A).
- For the 187 randomized patients: 21.9% for the RefStrategy, 29.9% for WGS-solo and 42.3% for WGS-trio (Fig. 2C).

When considering VUS+, the diagnostic yield increased by 13.9% in both groups, reaching 55.8% in the NeverTested group (Fig. 2B).

#### *Diagnostic yield by strategy in the NeverTested population*

For NeverTested patients, 3.1% were positive with the RefStrategy only (mostly fragile X diagnoses) and 27.6% with the WGS-trio only, with 55% of patients remaining unexplained (Fig. 3A and Additional file 2, Fig. S3). In the 187 randomized patients, 7.4% of cases were positive with RefStrategy only, 15.5% with WGS-solo only, and 62.6% remained negative (Fig. 3B and Additional file 2, Fig. S3). For WGS-solo compared to WGS-trio, 1% were positive for WGS-solo only (1 patient, 2 variants), 13% for WGS-trio only (24 patients, 28 variants) and 57% remained negative (107 patients) (Fig. 3C and Additional file 2, Fig. S3). All these variants are listed in the Additional file 3, Table S3.

The patient with WGS-solo+/WGS-trio− in fact carried two different heterozygous variants in *AUTS2* and *KMT2A*, which were each inherited from an asymptomatic parent, and which were finally both classified as variants of uncertain significance (VUS).

The 28 variants identified in WGS-trio but not retained in WGS-solo, across 24 patients, included:

- Eleven CNVs (7 losses, 4 gains): 7 Likely pathogenic/pathogenic CNVs (6 de novo, 1 maternally inherited), 3 CNVs with IPVE (one maternally inherited 16p11.2 gain, one de novo 16p11.2 loss, one paternally inherited 15q11.2q12 gain), and one VUS+ (de novo 17p13.2 gain).
- Seventeen SNVs: 15 Likely pathogenic/pathogenic SNVs (12 de novo, one homozygous variant inherited from both parents, two compound heterozygous variants inherited from each parent) and two VUS+ (one maternally inherited missense variant in *IL1RAPL1* in a male (X-linked) and one missense de novo variant in *KDM5C* in a female (X-linked)).

These variants were not kept in the WGS-solo analysis because information on their inheritance status (inherited from one of the parents or de novo) was essential for proper interpretation, especially for variants identified by WGS-trio classified as VUS+ and for CNVs with incomplete penetrance or variable expressivity (IPVE).

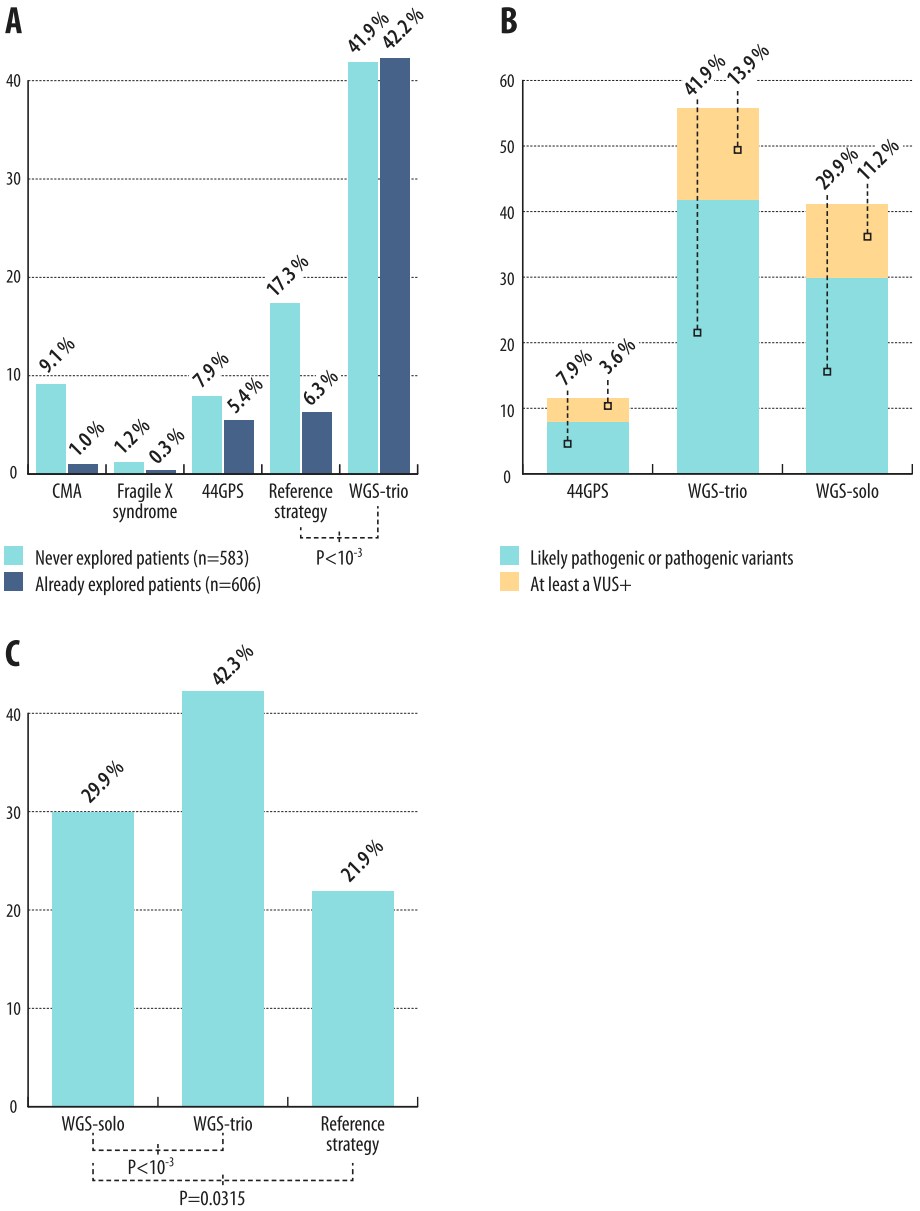


**Fig. 2** Diagnostic yields of the RefStrategy, WGS-trio, and WGS-solo in the 1239 ID patients. **A** McNemar tests between diagnostic yields of WGS-trio compared to the RefStrategy, the 44 ID genes panel (44GPS), fragile X expansion analysis and chromosomal micro array analysis (CMA) in already explored patients (AlreadyTested, dark blue bars) and never explored patients (NeverTested, light blue bars) ($p < 10^{-3}$). **B** Diagnostic yields of 44GPS, WGS-trio, and WGS-solo according to likely pathogenic/pathogenic variants (light blue bars) and VUS + (yellow bars), in NeverTested. **C** McNemar tests between diagnostic yields of WGS-solo compared to WGS-trio and to RefStrategy in the subgroup of 187 randomized NeverTested patients

(See figure on next page.)

**Fig. 3** Venn diagrams comparing the strategies in the NeverTested population. **A** Venn diagrams showing the positive diagnoses in the NeverTested group ($N = 583$): WGS trio (in blue) vs RefStrategy (in red). **B** Venn diagrams showing the positive diagnoses in the NeverTested randomized subgroup ($N = 187$): WGS solo (in green) vs RefStrategy (in red). **C** Venn diagrams showing the positive diagnoses in the NeverTested group ($N = 583$): WGS trio (in blue) vs WGS solo (in green). NeverTested: Never explored patients; RefStr: reference strategy; WGS: genome sequencing

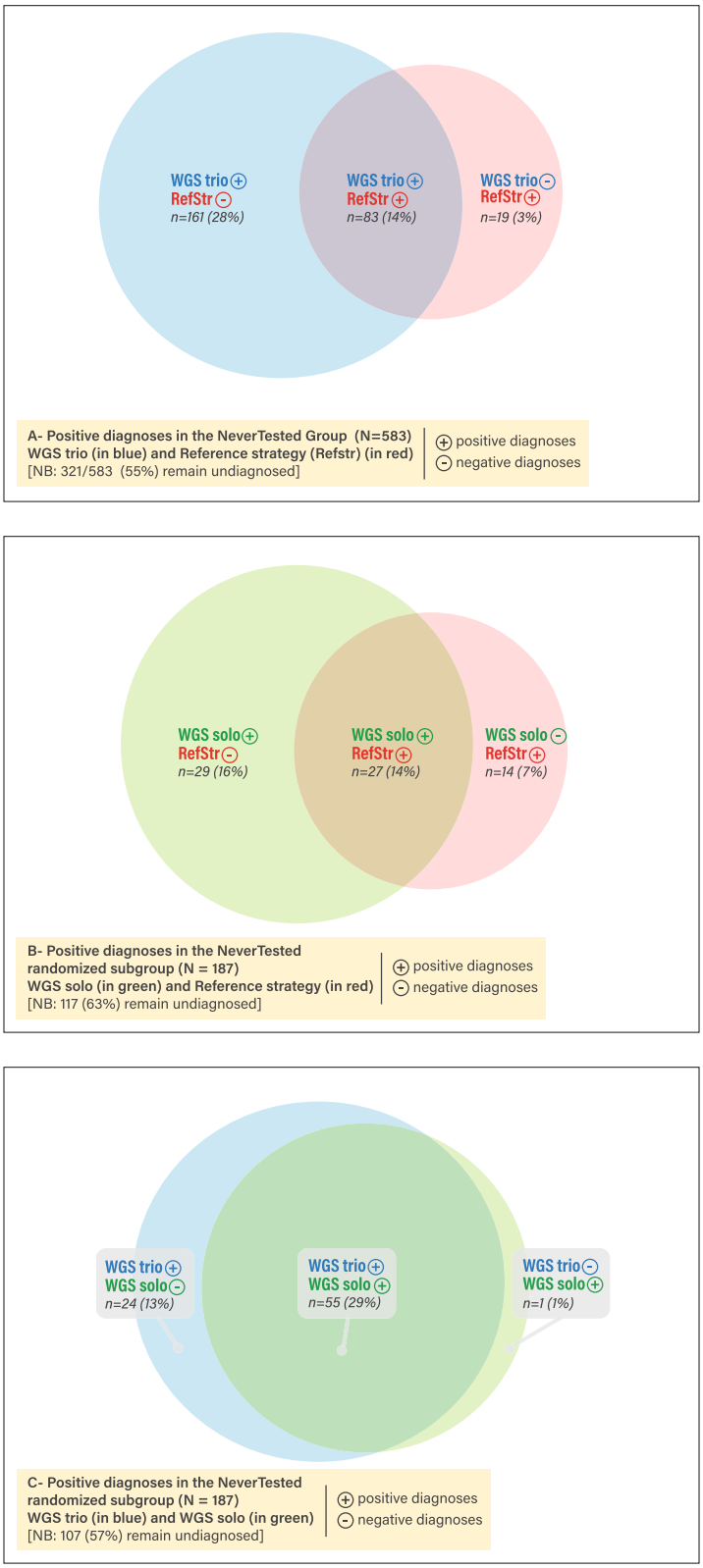


**Fig. 3** (See legend on previous page.)

### *Diagnostic rate of WGS-trio according to the type of genetic analysis previously performed*

In the AlreadyTested group, the WGS-trio diagnostic yield was 41% in case of negative ID gene panel prior to WGS and it dropped to 21% if patients had a negative WES prior to WGS-trio (see Additional file 2: Fig. S4).

### *RNU4-2 analysis*

A prompt re-analysis of the WGS-trio data made it possible to identify pathogenic de novo variants in the *RNU4-2* gene in seven patients (one NeverTested patient and six AlreadyTested patients). Among them, six individuals carried the recurrent *RNU4-2* pathogenic variant NC_000012.11:g.120729642_120729643insA, NR_003137.2:n.64_65insT (GRCh37) and one patient carried the pathogenic variant NC_000012.11:g.120729630_120729631insA, NR_003137.2:n.76_77insT (GRCh37) [73, 74, 76]. The clinical characteristics of these patients are summarized in Additional file 3: Table S4.

### Genetic heterogeneity and recurrent variants

When considering likely pathogenic/pathogenic SNVs, 442 were identified, involving a total of 231 genes, of which 62.6% were involved only once. The top three genes were (i) *DDX3X*, for which pathogenic SNVs were found in 10 female patients (5 AlreadyTested, 5 NeverTested), (ii) *MECP2*, with nine patients (8 females, 1 male) (5 AlreadyTested, 4 NeverTested), and (iii) *RNU4-2* with seven patients (6 AlreadyTested, 1 NeverTested). The list of the top 15 ID genes is shown in Fig. 4.

### Characteristics of the pathogenic variants

The types of likely pathogenic/pathogenic variants are detailed in Table 2.

Regarding the SNVs, 79% (350/442) were de novo. They involved autosomal dominant ID genes in 72% of cases (318/442), X-linked genes in 15% (65/442, including 53 de novo and 12 maternally inherited variants) and autosomal recessive genes in 13% (19 homozygous SNVs and 40 compound heterozygous SNVs/CNVs), including genes involved in inborn errors of metabolism for 11 patients including *GALT* (1), *MAN2B1* (2), *HSD17B4* (1), *PSPH* (1), *COQ4* (1), *ALDH5A1* (1), *MMACHC* (1), *FH* (1), *IVD* (1), and *PMM2* (1).

Overall, 506 patients were carrying at least one likely pathogenic/pathogenic variant accounting for their phenotypes. Among them, 3.9% had multiallelism (see Additional file 3: Table S5):

- Twenty-six patients carried a second variant in a distinct gene:
  - Additional likely pathogenic/pathogenic variant in 5 patients: 3 mild and 2 moderate ID.

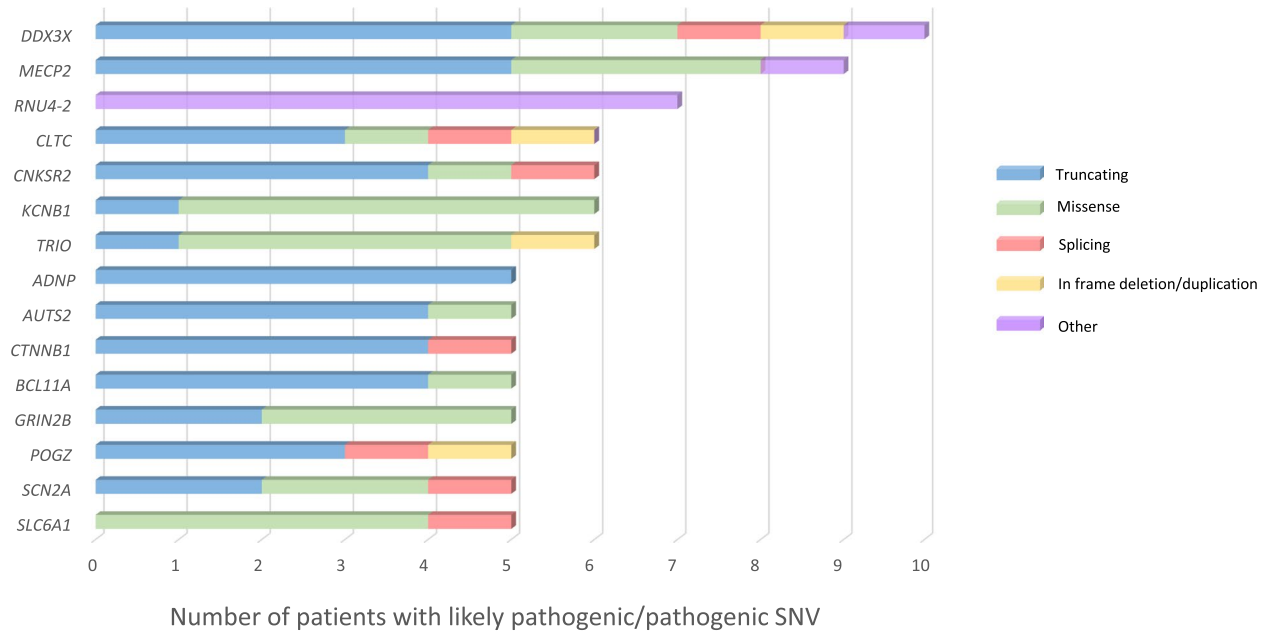


**Fig. 4** Top 15 ID genes involved in the DEFIDIAG cohort population (likely pathogenic/pathogenic SNVs). Blue bars represent truncating variants, green bars represent missense variants, red bars represent splicing variants, and yellow bars represent in frame deletions or duplications. All variants were de novo, except one *BCL11A* (2p16.1), one *TRIO* (5p15.2), one *CNKSR2* (Xp22.12), and one *MECP2* (Xq28) variant, which were all maternally inherited

**Table 2** Characteristics of the likely pathogenic/pathogenic variants and VUS + identified in the DEFIDIAG study (2020–2023)

| Variants | Likely pathogenic/pathogenic variants | VUS + | Total |
|---|---|---|---|
| **SNVs** | **442** | **171** | **613** |
| **Missense** | 214 | 116 | |
| **Nonsense** | 92 | 9 | |
| **Frameshift** | 83 | 14 | |
| **Splice variants** | 36 | 20 | |
| **Deletion/duplication (in-frame)** | 11 | 5 | |
| **Deep intronic** | 1 | 2 | |
| **Stop loss** | 0 | 2 | |
| **Start loss** | 2 | 0 | |
| **5′-UTR** | 0 | 2 | |
| **Synonym** | 0 | 1 | |
| **Other** | 3 | 0 | |
| **CNVs** | **79*** (63 de novo, 16 inherited) | **39** | **118** |
| **Gain** | 18 | 13 | |
| **Loss** | 61 | 26 | |
| **Size** | > 5 Mb: 22%<br>100 kb to 5 Mb: 55%<br>10 kb to 100 kb: 11%<br>< 10 kb: 12% | | |
| **SVs** | **10** | **0** | **10** |
| **Balanced translocation**** | 2 | 0 | |
| **Insertion** | 1 | 0 | |
| **Inversion** | 6 | 0 | |
| **Unbalanced translocation** | 1 | 0 | |

*CNVs* Copy number variants, *SNVs* Single-nucleotide variant, *SVs* Structural variant

* CNVs encountered in classically known syndromes were found recurrently [Pheland McDermid syndrome ($n=4$), Prader Willi/Angelman syndromes ($n=2$), 22q11.2 deletion syndrome ($n=2$), Jacobsen syndrome ($n=2$)] as well as classical CNVs with incomplete penetrance and variable expressivity including 16p11.2 deletion ($n=6$) (2 BP2-BP3 (distal), 3 BP4-BP5 (proximal) and one BP2-BP5 deletions) and duplication ($n=3$), 15q11.2 deletion (BP1-BP2) ($n=1$), 16p13.1 deletion ($n=2$), 1q21.1q21.2 deletion ($n=2$))

** Both reciprocal translocations were de novo and had breakpoints that interrupted the *RFX4* and *MBD5* genes

  - Or VUS + in 21 patients: 6 mild, 11 moderate, 3 severe, 1 profound ID.

- One patient—presenting with moderate ID—had a triple diagnosis (one de novo *MECP2* likely pathogenic variant, one inherited *COL1A2* likely pathogenic variant and one inherited pathogenic *SHOX* deletion; the latter did not explain the ID).

#### Identification of variants of interest in new ID genes

Among the patients with a VUS + or likely pathogenic/pathogenic variant, 16 carried a variant involving 16 different novel ID genes that had not yet been associated with ID as of the beginning of the DEFIDIAG study (12th March 2020), including *BAP1*, *BICRA* , *CDK16*, *CERT1*, *CTR9*, *CUL3*, *IRF2BPL*, *KDM4B*, *NUP85*, *SPEN*, *SPTBN1*, and *UNC79*. Among them, two patients, with variants in the *CDK16* and *CTR9* genes, were included in research papers that reported those as ID-associated genes for the first time [79, 80] (*CDK16* had been reported in 2018 as a candidate X-linked ID gene [81]).

#### Links to molecular pathways

To evaluate the functional representation of the 231 ID-associated genes (DEFIDIAG-ID genes) identified in the DEFIDIAG patient cohort, they were compared to those reported in the SysNDD database. The GO terms associated with each DEFIDIAG-ID gene were used to distribute them into the 32 SysNDD-defined functional categories [77]. DEFIDIAG-ID genes were significantly enriched in 7 pathways, the top 3 being: chromatin (137 patients), metabolism (134 patients) and synapse (102 patients)-related biological processes (Fig. 5).

## Discussion

The national DEFIDIAG study demonstrates in real-life routine medical genetic care that WGS-trio as the first-line diagnostic test for ID shows a significantly improved diagnostic yield (41.9%) compared to the national reference strategy. Diagnostic yields with WGS-trio were similar for NeverTested and AlreadyTested patients, at around 42%, and increasing to roughly 60% in both categories when VUS + were also considered. Moreover, WGS-trio led to a causal diagnosis in AlreadyTested patients with previously negative WES (21%). All these observations are in line with other studies and confirm the utility of performing WGS as a major diagnostic test for ID, among other neurodevelopmental disorders (NDD) [1, 3, 5, 40, 82, 83]. It might seem surprising at first glance that the diagnostic yield of WGS-trio was similar in the NeverTested and AlreadyTested groups, as one would expect a lower yield in the AlreadyTested group, given that these patients had already undergone prior genetic testing. However, this may be partially explained by the way patients were recruited in the two groups. Indeed, those included in the AlreadyTested group had most often been followed for several years for unexplained severe ID — which accounts for the higher mean age in this group — with a clinical presentation that led the expert clinical geneticist to strongly suspect an underlying genetic etiology. Despite the prospective design of our study, we could not avoid all classification bias. Indeed, since the patients were included when they presented at the clinic, regardless of whether they were coming for a first visit (and thus, were Never Tested) or whether they were Already Tested, one potential limitation is that, sometimes, we had poor accessibility to such documents or results, if the patient had consulted in many hospitals. However, through thorough monitoring, we identified some deviations—up to 19% in the NeverTested group, particularly for fragile X identification or CMA (17%). These tests being negative (otherwise they would not have been included), it may have led to a slight overestimation of differences between WGS-trio and the reference strategy. However, given the observed differences, excluding those patients would not change the conclusions.

Around 28% of patients with likely pathogenic/pathogenic variants were identified only by WGS-trio and were missed by the standard routine genetic analyses in the national RefStrategy (Additional file 2: Fig. S5). This underlines the added value of WGS as an "all-in-one test," with enhanced detection of (1) variants in genes that are not included in the 44GPS panel; (2) variants located in non-coding regions (such as the recent non-coding spliceosomal *RNU4-2*); (3) variants in regions poorly covered by WES; (4) SVs undetectable by CMA, including complex and balanced chromosomal rearrangements with possible gene disruption mechanisms or position effects [3, 73, 76, 84].

The DEFIDIAG study identified 3% (19/583) of NeverTested patients who had a positive RefStrategy result,

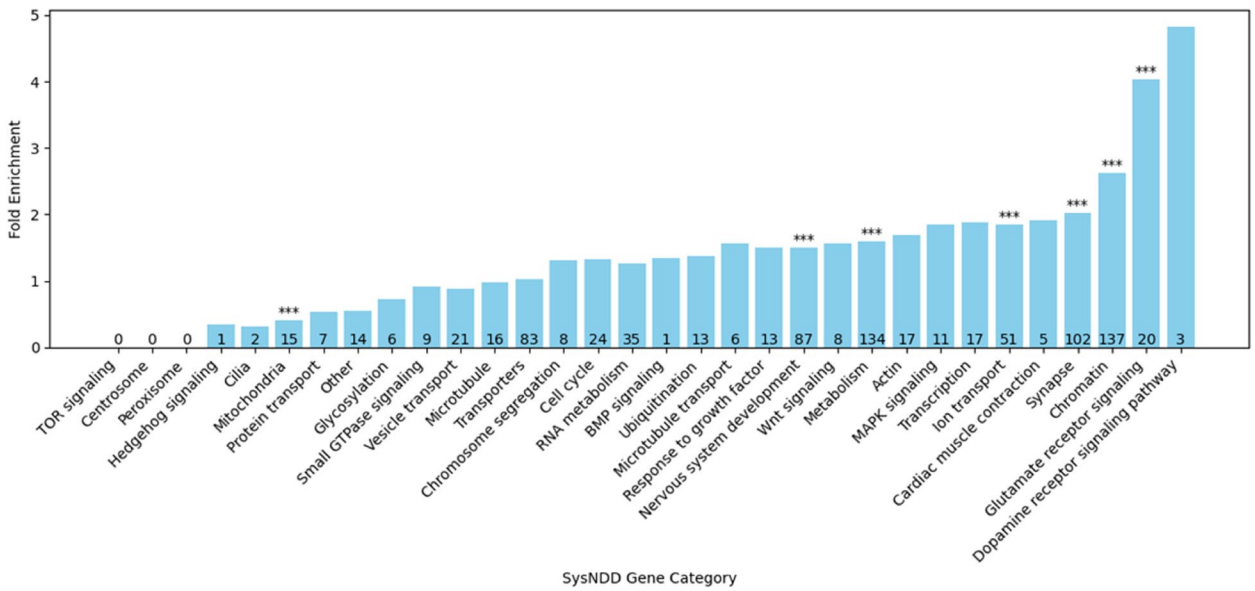


**Fig. 5** Representation of gene enrichment in DEFIDIAG compared with gene categories in the SysNDD database. Bar diagrams show enrichment of ID-DEFIDIAG-ID genes in each indicated functional category against the SysNDD ID-genes as background. The total number of DEFIDIAG genes per category is displayed in the respective bar. The asterisks indicate statistically significantly enriched categories (Fisher test, Benjamini-Hochberg; ∗ adjusted $p < 0.05$, ∗ ∗ adjusted $p < 0.01$, ∗ ∗ ∗ adjusted $p < 0.001$.). Adapted from SysID database [69]

but a negative WGS-trio result (Fig. 3A and Additional file 2, Fig. S3). The main cause was fragile X syndrome. Future implementation of long-read WGS and/or optical mapping will improve detection of GC-rich abnormal repeats (such as for fragile X syndrome) and other regions enriched with repeats (telomeres, centromeres). Indeed, short-read sequencing is known to be limited for detecting breakpoints occurring in repeated sequences, in particular duplicons and alpha satellites [85–88]. For these reasons, although the tools used for genome data interpretation are becoming increasingly efficient at detecting certain repeat expansions, targeted testing for Fragile X syndrome remains necessary alongside genome sequencing at this time.

WGS-solo and WGS-trio gave similar results in 68.8% (55/80) of positive patients meaning that, although less powerful, WGS-solo analysis can be of clinical utility and performs better than the RefStrategy (29.9% *vs* 21.9%, respectively; $p = 0.0315$) (Fig. 2C). In real-life clinical practice, unavailability of parental DNA (one parent or both) is not uncommon, and imposes WGS-solo testing (or *duo* WGS, an intermediate situation not explored here) as the only way to obtain a diagnosis, in an effort to provide access to diagnosis for all patients affected by ID. Overall, DEFIDIAG shows that WGS-solo can lead to a diagnosis in around 1/3 of cases.

A total of 13.9% of VUS + were detected in both AlreadyTested and NeverTested groups, most of them requiring further investigations (usually in a research laboratory setting unavailable in a hospital) to validate the variant as pathogenic. For example, a study performing WGS in 2183 probands with a broad spectrum of RD, reported that 14% of the diagnoses were made thanks to automated hospital approaches combined with research settings for validation of non-coding, structural and poorly covered region variants [1]. Thus, investing time and funding into resolving VUS + is a high clinical need and may require gene-tailored research investigations of prime importance to deliver a diagnosis in a care context.

Overall, as expected, de novo variants were the most frequent pathogenic variants, as reported in many studies [25, 83, 89–91].

Multiple molecular diagnoses were observed in 3.9% of patients disclosing multiallelism (defined by different diseases occurring in a given patient and proven by genetic testing) (See Additional file 3: Table S5). The DEFIDIAG multiallelism rate is consistent with other WGS studies (1.8 to 7.1%) [92, 93]. Multiallelism can explain complex phenotypic presentations (such as unexplained overlap of syndromic phenotypes that can at first be discordant, and misleading for clinical diagnosis). WGS is of high clinical utility, especially when patients present with a discordant or unexplained association of phenotypes that do not fit the classical description of a known genetic condition. Moreover, the occurrence of multiallelism in DEFIDIAG cases raised the question of increased ID severity phenotypes in a given patient, as each diagnosis was independently associated with ID. This was not the case, as, among the 27 patients with multiallelism, the ID was moderate in 14 patients, severe in three patients and profound in one patient.

The DEFIDIAG study revealed a total of 231 genes recognized as causative and delivered to the clinician following the multidisciplinary meeting. The top three most prevalent genes were *DDX3X*, *MECP2*, and *RNU4-2*. For *DDX3X*, 10 female patients (5 AlreadyTested, 5 NeverTested) were found to carry a de novo likely pathogenic/pathogenic variant. This is in line with *DDX3X*, a well-known X-linked gene involved in the Snijders-Block syndrome (MIM 300958), affecting females more than males [94, 95]. The second causative gene was *MECP2*, also a classic X-linked syndromic ID, with eight female patients and one male patient (5 AlreadyTested, 4 NeverTested) carrying a pathogenic SNV, and one female patient carrying a de novo complex SV involving *MECP2*. All the variants identified in females were de novo, in line with the classical description of Rett syndrome (MIM 312750) [96, 97]. The top two genes are in accordance with other published large ID cohorts, in which *DDX3X* and *MECP2* emerged among the top five most frequent ID genes [5, 94, 98] (Fig. 4).

The third causative gene was the recently described autosomal *RNU4-2* non-coding gene reported in seven patients (6 females and 1 male) (all except one were AlreadyTested) who presented with ID ranging from mild (2 cases), to moderate (1 case), severe (3 cases), and profound (1 case). Overall, as described in the recent literature, the *RNU4-2* subgroup disclosed variable syndromic phenotypes with facial dysmorphism and hypotonia as the main features [73–76]. The immediate availability of DEFIDIAG cohort WGS data is an added value for rapid re-analysis in case of novel gene identification by other groups, as proven by the rapid investigation performed for *RNU4-2*. The seven patients reported to carry pathogenic de novo *RNU4-2* variants, represent a prevalence of 0.59%, which is in accordance with recently reported rates of 0.41% [76], 0.50% [73, 75], and 0.61% [74].

The numerous remaining causative genes occur in fewer patients or even a single patient and represent a plethora of reported ID genes with a high rate of de novo variants.

Overall, it is now well recognized that WGS significantly reduces the diagnostic odyssey of patients. The duration from the first clinical contact in the center of expertise to the consultation to deliver the DEFIDIAG

results was on average 8.6 years, regardless of the result (Fig. 6). Regarding the evaluation of time efficiency—although this was not one of the main aims of this study—the single-test WGS approach eliminates the waiting period associated with the sequential completion and reporting of multiple genetic analyses—such as CMA and gene panels—which can still take several months or even over a year. Furthermore, implementation of a single-test strategy is particularly impactful in scenarios requiring urgent genetic counseling, such as ongoing pregnancies. Identically, a fast-track diagnostic pathway has been established within the PFMG2025 framework for critically ill children admitted to pediatric intensive care units. In such urgent situations, the benefit of a rapid and comprehensive diagnostic test is obvious, with direct and significant implications for therapeutic management [99, 100]. Regarding the evaluation of the cost effectiveness of a unique test approach in the context of DEFIDIAG, a health economics study is underway (by Binquet and collaborators). Several recent studies have demonstrated that, compared with current clinical practice, simplified access to WGS in patients with suspected genetic conditions—including developmental anomalies and ID—shortens diagnostic wandering, while reducing diagnostic interactions with the healthcare system and thus cutting costs (fewer hospitalizations, unnecessary complementary tests, and specialized consultations) [82, 101–103]. Furthermore, the cost of WGS has substantially decreased over the last decade, which, combined with its diagnostic superiority over WES in detecting variants in noncoding regions, should lead to increased use in clinical settings [104].

The DEFIDIAG study also showed the clinical utility of mandatory multidisciplinary meetings for comparing the medical geneticist's diagnosis with the molecular biologist's results. These meetings have proven to be highly informative to confirm diagnoses or to exclude VUS that were not appropriate. Classical causes of syndromic ID are usually easily recognizable on examination by an expert clinical geneticist (and therefore such cases were a priori excluded from this study, as specific testing is available). However, the DEFIDIAG study identified several patients with so-called classical syndromes (Table 3). This highlights the power of WGS in detecting such missed cases in real-life clinical situations where recognition of classical syndromes may fail due to the phenotypic variability, even with high level clinical expertise.

In addition, WGS enabled diagnosis of rare inborn errors of metabolism (IEM) including well-known conditions that could have been diagnosed by biochemical analyses, such as galactosemia (MIM 230400), isovaleric acidemia (MIM 243500), CDG type Ia (MIM 212065), and methylmalonic aciduria (MIM 277400), but also rarer disorders for which affected patients may not show specific biochemical defects, like Coenzyme Q10 deficiency-7 (MIM 616276) [105]. More than 116 forms of

(See figure on next page.)

**Fig. 6** Illustrative cases showing patients' diagnostic odyssey. Patient 1 is a 29-year-old male with severe ID, seizures with continuous spike–waves during slow sleep EEG pattern, facial dysmorphic features (**a** at age 10 years, **b** and **f** at age 26 years), and mildly hypoplastic nails (**e**). The patient underwent testing for Fragile X syndrome, CMA, and a 556 ID genes panel, all results were negatives. After 22 years of diagnostic wandering, the DEFIDIAG study identified a de novo likely pathogenic heterozygous missense variant in *POU3F3* (Chr2(GRCh37):g.105473206 T > C; NM_006236.2(POU3F3):c.1238 T > C; p.(Ile413Thr)). This result highlights the value of WGS compared to ID gene panels, which, although regularly updated, include a limited number of genes. Patient 2 is a 17-year-old male affected by severe ID, autism spectrum disorder, early epilepsy followed by neurological regression, facial dysmorphism (**c**, **d**) and short distal phalanges (**g**). Brain MRI showed thickening of the corpus callosum (arrow) and widening of the vermian sulcus (**h**). Both CMA and a 207 genes panel targeting epilepsy and cortical malformations yielded negative results. After 17 years of diagnostic wandering, the DEFIDIAG study made it possible to identify a de novo pathogenic chromosome 5 paracentric inversion involving *MEF2C* (Seq[GRCh37] inv(5)(q14.3q14.3), NC_000005.9:g.88090783_88605087inv) {ISCN 2020}. This clinical case clearly illustrates the superiority of WGS in identifying structural variants. Patient 3 is a 5-year-old female patient presenting with ID and overall developmental delay predominantly affecting language, behavioral and social interaction difficulties, recurrent infections, chronic constipation, and visual impairment. She presents with distinctive craniofacial features, including a prominent forehead and a high anterior hairline (**i**, **j**), as well as broad and short hands with tapered fingers and enlarged halluces (**k**, **l**). She was enrolled in the DEFIDIAG study, and WGS trio identified a heterozygous de novo pathogenic intragenic inversion in the *ADNP* gene (Chr20(GRCh37):g. 49515761_49525309inv, NM_001282531.3:c.–89–3923_201 + 2793 inv). This structural variant encompasses exons 3 to 5, involving the two first coding exons with the initiation Met1. RNAseq experiment showed a splice skipping of the inversed exons and in silico analysis suggested that several initiating ATGs would lead to the failure of any in-frame rescuing translation, because of out-of frame ATGs, resulting in haploinsufficiency. Since this inversion is undetectable by exome sequencing, this case emphasizes the added value of whole genome sequencing [110]. Patient 4 is a 9-year-old female born with intrauterine growth restriction and a velar cleft. She achieved independent walking at 23 months, and first spoken words emerged at 20 months. She has since developed mild ID, associated with microcephaly (– 3 SD) and significant anxiety. Feeding remains problematic due to pronounced food selectivity, notably with a consistent refusal to consume fruit and vegetables. She presents with facial features (**m**, **n**) including epicanthal folds, hypertelorism, tubular nose with broad and prominent nasal bridge, and large dysplastic ears. The examination of the extremities showed mild, nonspecific morphological anomalies, including slightly low-set thumbs (**o**, **p**). 22q11 FISH analysis and CMA were negative and WGS-trio identified a heterozygous de novo pathogenic nonsense variant in *TLK2* (Chr17(GRCh37):g.60642437C > T, NM_006852.6(TLK2):c.907C > T p.(Arg303*))

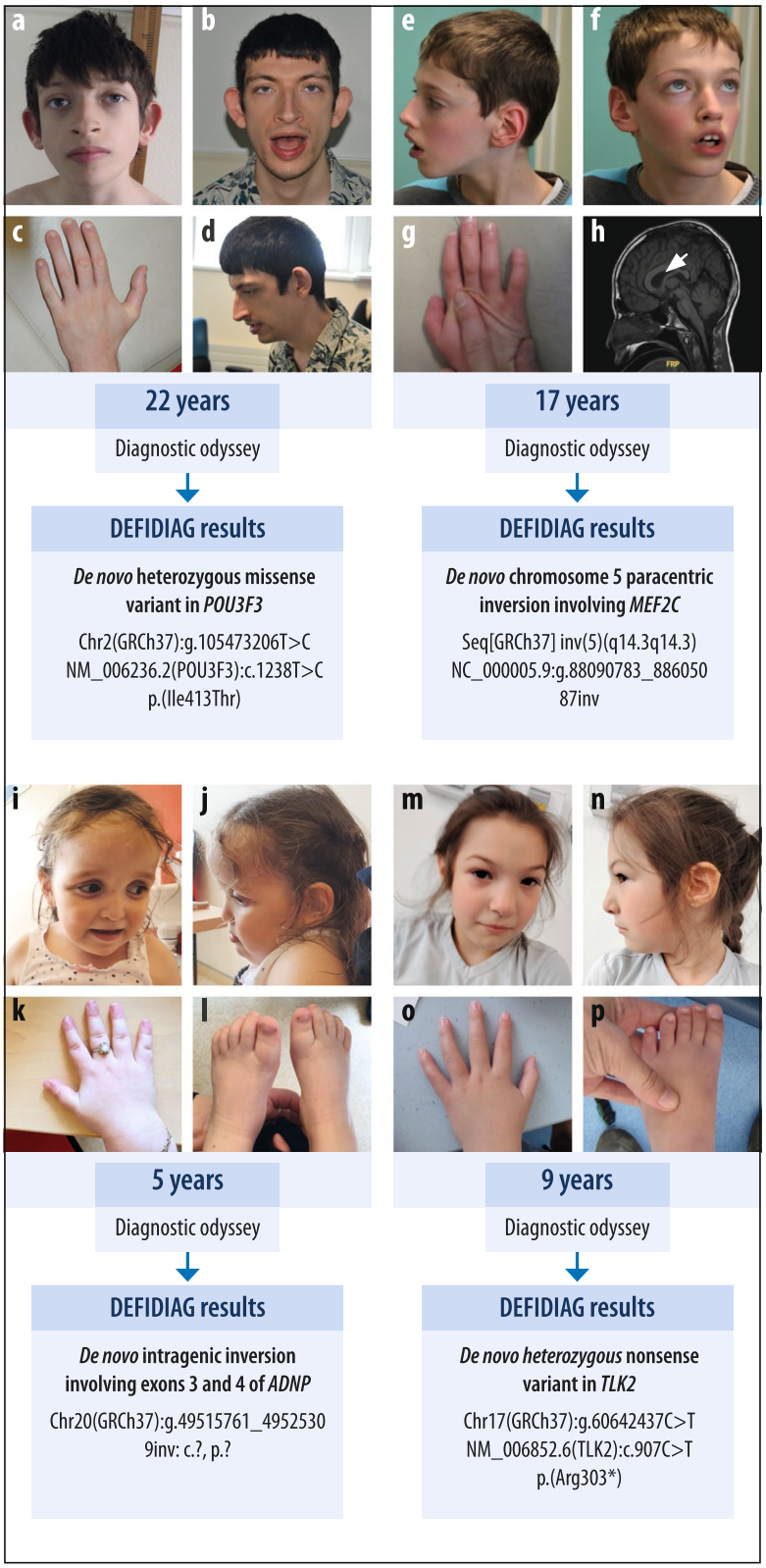


**Fig. 6** (See legend on previous page.)

**Table 3** Patients from the DEFIDIAG study diagnosed with a classical well-known syndrome

| Syndrome | MIM | *N* |
|---|---|---|
| **Rett syndrome** | 312,750 | 8 |
| **Angelman syndrome** | 105,830 | 3 |
| **Malan syndrome** | 614,753 | 3 |
| **22q11.21 microdeletion syndrome** | 188,400 | 2 |
| **Noonan syndrome** | 163,950 | 2 |
| **Prader Willi** | 176,270 | 1 |
| **Sotos syndrome** | 117,550 | 1 |
| **Coffin Lowry syndrome** | 303,600 | 1 |
| **Cornelia de Lange syndrome** | 122,470 | 1 |

metabolic ID have been identified as treatable, mostly by nutritional treatments adapted to metabolic disorders or pharmacological therapies or enzyme replacement therapy [106]. In this respect, among the 231 genes recognized as causative in the DEFIDIAG study, twelve can be considered as actionable (*PSPH*, *MAN2B1*, *SLC6A8*, *MMACHC*, *ALDH5A1*, *GRIN1*, *GRIN2A*, *GRIN2B*, *PMM2*, *GALT*, *COQ4*, and *IVD*), with responsiveness sometimes being genotype-dependent [107]. This is of high clinical relevance, since these rare diseases have mostly no recognizable clinical phenotype and WGS may accelerate delivery of a specific therapeutic action, enhancing WGS as an improved precision medicine approach.

Likewise, in several DEFIDIAG study patients with seizures, WGS revealed epilepsy-related conditions with potential specific therapeutic approaches according to the gene involved, such as *SLC6A1*, *SCN8A*, *SCN2A*, *KNCQ2*, *CDKL5*, *GRIN2A*, *GRIN2B*, *STXBP1*, *CACNA1A*, or *PCDH19* [108, 109]. Obtaining such diagnoses is crucial, in particular in very young children, to quickly start, or, on the contrary, avoid specific anti-epileptic drugs, sometimes depending on the kind of variants (gain or loss of function). For instance, sodium channel blockers and ketogenic diet are known to lead to favorable response in *KCNQ2* and *SLC2A1*-related syndromes, respectively, while sodium channel blockers and valproic acid tend to worsen seizures in Dravet syndrome and *POLG* epileptic encephalopathy, respectively, in which they should thus be avoided [108, 109].

During the timeframe of the DEFIDIAG study, variants were identified in 16 novel ID genes (defined as having been unpublished before the first patient inclusion in March 2020), including at least two novel ID genes for which DEFIDIAG patients were added to collaborative international initiatives [79, 80].

Currently, according to the SysNDD database [10] dedicated to genes and pathways involved in NDD, nearly 1700 genes are contributing to around 1800 ID conditions (syndromic or non-syndromic). These genes encode for proteins involved in numerous biological and metabolic pathways, including synaptic function, epigenetic regulation, intracellular transport, transcription or embryonic brain development. By comparing the 231 DEFIDIAG genes with likely pathogenic/pathogenic variants to the SysNDD database, seven SysNDD-related pathways were enriched in the DEFIDAG cohort illustrating the broad spectrum of biological pathways involved in ID: mitochondrial, nervous system development, metabolism, ion transport, synapse, chromatin, and glutamate receptor signaling (Fig. 5), which were also mainly represented in other publications [77, 83].

In contrast to the improved diagnostic yield, the DEFIDIAG results underline that around 50% of ID patients still remain undiagnosed. Overall, this is in line with the range of ID patients still devoid of any genetic diagnosis as currently reported in the literature [74, 110]. As the short-read WGS data is easily accessible for re-analysis with improved bioinformatic tools, subsequent re-analysis of undiagnosed patients may uncover pathogenic variants months or years after the first analysis, and can increase the diagnostic yield up to 20% [111–113]. Re-analysis includes reappraisal of variants previously identified but not initially considered as disease-causing, detection of non-coding variants or complex structural anomalies [114–116]. Several authors thus stress the importance of clinical expertise and clinician-biologist interactions, particularly when re-analysis is guided by a strong clinical suspicion after a first negative analysis [29]. This is congruent with the DEFIDIAG study, with regular MDMs bringing together the expertise of clinicians and biologists (and repeated if necessary for a given patient). As DEFIDIAG is a pilot research study linked to the PFMG2025 initiative, dataset harmonization is planned through the CAD (Collecteur Analyseur de Données—data analyzer & collector [117]) to enable easier future access to the data, and to facilitate data access for both research and diagnostic purposes, including the re-analysis of data in unsolved cases.

Unsolved cases can also point to overlooked non-genetic causes, unknown genetic determinants or complex genetic causes fitting the concept of missing heritability [118]. Regarding the unsolved cases of the DEFIDIAG cohort (262 NeverTested patients and 272 AlreadyTested patients), various alternative genetic causes could be hypothesized, for example: epigenetic modifications, variants in non-coding regulatory regions, small open reading frames (smORFs) [119] or the different snRNA genes [75, 120], long tandem repeat

expansions, uniparental disomies, gene-pseudogene inversion [121, 122] as well as mobile element insertions. Unsolved cases may benefit from the avenues for future research beyond re-analysis. Following the revolution of WGS, long-read sequencing holds promise to increase yields by at least 10%, and multi-Omics approaches are blooming, especially RNA sequencing approaches. Proteomics and epigenetics studies should also increase the yields as strategies that could reach routine care in the near future. Functional investigations for novel genes will enjoy improved access to models including in vitro via iPSCs and neuronal modeling as well as for various in vivo models [87, 88, 123–128].

## Conclusion

The demonstration by the DEFIDIAG study of the added diagnostic value of WGS paves the way to significantly reducing the diagnostic odyssey and enhancing precision medicine. Since the end of inclusions in the DEFIDIAG study, further French patients with ID have benefited from the PFMG care pathways, with a median delivery time of 134 days, and delivery of 5651 reports (positive or negative) over the last 3 years [100]. At the level of the French population, this proven added diagnostic value of first-line genome sequencing supports the DEDIFIAG consortium's recommendation to implement genome sequencing as the first-tier test for individuals with ID — and, more broadly, for all patients with rare diseases — in order to significantly reduce the diagnostic odyssey and provide access to precision medicine for all.

### Abbreviations

| | |
|---|---|
| ACMG | American College of Medical Genetics and Genomics |
| AlreadyTested | Already explored patients |
| ANPGM | Association Nationale des Praticiens de Génétique Moléculaire |
| AR | Autosomal recessive |
| BAM | Binary Alignment and Map |
| bp | Base pairs |
| CAD | Data analyzer collector /Collecteur Analyseur de Données |
| CEA-CNRGH | Commissariat à l'Énergie Atomique—Centre National de Recherche en Génomique Humaine |
| CMA | Chromosomal microarray analysis |
| CNV | Copy number variant |
| DGV | Database of Genomic Variants |
| DIN | DNA integrity number |
| e-CRF | Electronic Case Report Form |
| WES | Whole exome sequencing |
| GATK | Genome Analysis Toolkit |
| GO | Gene Ontology |
| WGS | Whole genome sequencing |
| WGS-trio | Whole genome sequencing in trio |
| WGS-solo | Whole genome sequencing in solo |
| gVCF | Genomic Variant Calling Format |
| HGMD | Human Gene Mutation Database |
| ID | Intellectual disability |
| IEM | Inborn errors of metabolism |
| IGV | Integrative Genomics Viewer |
| Indel | Insertion / deletion variant |
| IQR | Inter-quartile range |
| MIM | Mendelian Inheritance in Man catalog entry |
| OMIM | Online Mendelian Inheritance in Man |
| NDD | Neurodevelopmental disorder |
| NeverTested | Never explored patients |
| NGS | Next-generation sequencing |
| PFMG | Plan France Médecine Génomique 2025 |
| PNMR | National plan for rare diseases / Plan National Maladies Rares |
| SNV | Single-nucleotide variant |
| SRY | Sex-determining region of Y chromosome |
| SV | Structural variant |
| SysNDD | Database of neurodevelopmental disorders |
| RD | Rare diseases |
| RefStrategy | Minimal reference strategy |
| UH | University hospital |
| VABS | Vineland Adaptive Behavior Scale |
| VCF | Variant Calling Format |
| WAIS | Wechsler Adult Intelligence Scale |
| WISC | Wechsler Intelligence Scale for Children |
| WG | Weeks of gestation |
| WPPSI | Wechsler Preschool and Primary Scale of Intelligence |
| XL | X-linked |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13073-025-01527-4.

Additional file 1: List of the 44 ID genes included in the 44GPS panel.

Additional file 2: Supplementary Figures. Fig. S1. Flow Chart of the DEFIDIAG study. Fig. S2. DEFIDIAG study timeline. Fig. S3. Diagnostic yields by comparing the strategies in the NeverTested population. Fig. S4. Diagnostic yield of genome sequencing-trio (GST) in already explored patients (AEP) according to the previous genetic analyses performed. Fig. S5. Percentage of causal diagnoses by GST alone in the never-explored patients (NEP).

Additional file 3: Supplementary Tables. Table S1. Inclusion and exclusion criteria of the DEFIDIAG Study.Table S2. Diagnostic yields of GWS-trio vs RefStrategy according to age, ID severity and clinical subgroups, in NeverTested and AlreadyTested patients. Table S3. List of the variants that were exclusively detected by WGS-trio or WGS-solo in NeverTested patients. Table S4. Clinical characteristics of the seven patients carrying a de novo pathogenic variant in RNU4-2. Table S5. Patients from the DEFIDIAG study with multiallelism.

Additional file 4: Command lines and related software descriptions used for the DEFIDIAG study.

Additional file 5: Study Protocol.

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**Authors' contributions**

Clinical expertise and review of the paper: SEC, SH, CQ, MR, HM, DG, BI, AG, CG, GL, MW, CO, RC, TB, DB, AMG, IM, GV, PM, AP, AG, CC, MV, VM, CM, PC, SS, PE, DL. Biological expertise and review of the paper: GN, PSV, FL, KC, BK, TC, JMDSA, VM, GB, FTMT, HS, CP, JT, NC, LJ, AP, VH. Management of the study: CD, AB, FN, MB, MLHA, AS, NS. Conception of the study, pilot committee follow up, senior contribution and revision of the paper: BG, CL, LF, DS, DH, SO, PN, CSB, SL, JFD. General supervision, conception of the study, management of the study and writing of the paper: HD, CB, SEC. Pilot committee leaders: HD, CB.

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**Data availability**

The study protocol, statistical analysis plan, and informed consent form, will be made available (on publication) on request to the corresponding author or to the sponsor's representative ( promoteur.inserm@inserm.fr ) without restriction.

All requests for the access to the DEFIDIAG study data will be considered by the DEFIDIAG trial steering committee.

It should be noted that for participants who provided consent, data (clinical data excluding medical images + genetic data—VCF and BAM files) will be transmitted to and stored by the "CAD" (*Collecteur Analyseur de Données*—data analyzer & collector) of the French initiative for genomic medicine PFMG2025 (https://pfmg2025.fr/le-plan/collecteur-analyseur-de-donnees-cad/), for potential re-use by other researchers including those not involved in the present study.

Indeed, PFMG2025 was designed to ensure continuity between care and research, with the aim of promoting data sharing at both national and international levels, and enabling researchers to reuse data. Secondary use of the data for research will be possible within the national IT and data infrastructure currently under development (CAD), which will serve as a centralized access point for PFMG2025 data. In the long term, the CAD will offer high-performance computing capabilities within secure data processing environments. Pathogenic variants that were notified to patients in clinical care will be deposited in ClinVar.

The reuse of PFMG2025 data for research is aligned with an open science approach. Access to data is intended to be as broad as possible, while guaranteeing data security and adhering to defined scientific and ethical standards ( PFMG2025 summary document ).

The Scientific and Ethics Committee (CSE) of the CAD is responsible for ensuring that all research projects requesting access comply with these criteria. A specific procedure has been established (Accès aux données – PFMG 2025). The project leader must contact the CAD support desk and submit an application (PFMG2025-application file-CAD).

The CAD will first assess the admissibility and feasibility of the project. Subsequently, the CSE will assess whether the project complies with the data access criteria. Based on a collegial review, the CSE may issue one of the following decisions:

- a favorable opinion,
- a favorable opinion subject to project maturity,
- or an unfavorable opinion.

If the opinion is favorable subject to project maturity, the project receives methodological support from CAD teams, enabling resubmission for a new assessment by the CSE. If a favorable opinion is ultimately issued, and once regulatory authorizations have been obtained, a dedicated secure

environment will be created within the CAD infrastructure to grant access to the requested PFMG2025 data.

## Declarations

### Ethics approval and consent to participate

The DEFIDIAG study was conducted in accordance with the Declaration of Helsinki and its amendments. The protocol was approved by the Ethics Committee Sud Méditerranée I and the French data privacy commission (CNIL, authorization 919361).

### Consent for publication

Written informed consent to participate was obtained from each participant. Written parental consent was obtained for the publication of patient photographs included in this article.

### Competing interests

The authors declare no competing interests.

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## Publisher's Note