



Diagnostic yield of clinical exome sequencing in 868 children with neurodevelopmental disorders

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ABSTRACT

Next generation sequencing has revolutionized the diagnostic approach for patients with neurodevelopmental disorders (NDDs), yields are however highly variable depending on the patient's phenotype. It is often challenging to predict which indications are likely to lead to a molecular diagnosis and which will benefit less from genetic testing.

To identify phenotypic characteristics associated with higher diagnostic yields we conducted detailed phenotyping of a cohort of 868 children with NDD, who underwent clinical exome sequencing between 2016 and 2021.

A molecular diagnosis was reached in 27 % of cases. Significantly higher yields of respectively 34 % and 32 % were observed in patients with intellectual disability (ID) or global developmental delay (GDD). Autism spectrum disorders (ASD) were less likely to result in a molecular diagnosis with a diagnostic yield of 16 %. Additional factors linked to higher yields included female gender, the presence of minor dysmorphic features — particularly involving the face, extremities, ears, eyes, and hair — and a syndromic phenotype.

Additional CNV calling in a subset of 438 patients which consented to reanalysis of sequencing data added 1.5 % to diagnostic yield.

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1. Introduction

Neurodevelopmental disorders (NDDs) are a highly heterogeneous set of disorders, characterized by developmental deficits that lead to impairments of personal, social, academic or occupational functioning. They may also include conditions such as epilepsy and cerebral palsy. NDD are common, affecting up to 1 in 6 children when milder forms are taken into account, with most of them being suspected to have a genetic basis (Boyle et al., 2011).

Patients affected by NDD, especially when severe and syndromic, often endure what is described as a “diagnostic odyssey”, undergoing multiple invasive diagnostic tests, before reaching, if ever, a definitive diagnosis. Establishing a clear genetic diagnosis is critical for multiple reasons. It might open the door for an etiology-specific treatment, help to detect and treat associated comorbidities and to limit potentially harmful treatments or invasive diagnostic procedures. It is also essential for genetic counseling and to assess the risk of recurrence (Savatt and Myers, 2021).

NDDs are characterized by a high genetic heterogeneity, with over 1800 definitive NDD genes being reported in SysNDD (<https://sysndd.dbmr.unibe.ch/>). They are thus generally best investigated using agnostic next-generation sequencing (NGS) approaches, which explore a large number of potential candidate genes in parallel (Srivastava et al., 2019). Due to their high diagnostic yield, NGS sequencing approaches are currently recommended as a first tier investigation for patients with NDD, even before molecular karyotyping (Srivastava et al., 2019).

Clinical exome analysis (also known as partial exome, medical exome or mendeliome analysis) consists in sequencing all genes which are known to be associated with mendelian diseases. Advantages of this approach are its shorter analysis time, lower sequencing cost and better sequencing depth coverage, at the expense of exome completeness which limits the potential benefits of data reanalysis over time (Saudi Mendeliome Group, 2015). Diagnostic yield of clinical exome panels in the literature is heterogeneous ranging from 20 to 34 % in cohorts of NDD patients (Gieldon et al., 2018; Martinez-Granero et al., 2021). Performance of NGS in NDD is highly dependent on the patient’s phenotype, with a recent meta-analysis showing yields ranging from 16 % for autism spectrum disorder (ASD) patients up to 28 % for patients presenting with intellectual disability (ID) (Stefanski et al., 2021).

Indications for genetic testing are not always clear for primary care physicians and pediatricians. Developmental delay, for example, is often not recognized as a potential indication for genetic testing, despite its high potential for a diagnosis (Truong et al., 2021). A better understanding of the expected diagnostic yield for different phenotypes might be useful to ensure that patients with a probable genetic etiology are tested, and to manage expectations concerning the probability to find a genetic diagnosis.

The main variant types detected by NGS sequencing approaches are single nucleotide variants and small indels. Nowadays Copy Number Variation (CNV) calling algorithms also allow detection of larger structural variants using short-read NGS data. This approach has been shown to increase diagnostic yield by 1–2 % (Marchuk et al., 2018; Testard et al., 2022) and is able to identify exon-sized CNVs which are often too small to be detected using conventional array comparative genomic hybridization (aCGH) analysis (Yoon et al., 2009).

In this work we set out to determine the diagnostic yield of an in-house designed clinical exome panel in a cohort of 868 children with NDD. This work adds to the existing literature on clinical exome panels, which has mostly focused on ready-made exome panels, while in-house designed and regularly updated approaches have been rarely reported. We also aim to provide detailed phenotype descriptions of our patients to assess which secondary features are predictive of higher diagnostic yields in NDD. Furthermore we aim to assess the added diagnostic yield of read-depth based CNV calling using the CoNVaDING algorithm (Johansson et al., 2016) on patients for whom mendeliome sequencing did not provide a molecular explanation to their symptoms.

2. Methodology

2.1. Study cohort selection

The study cohort is based on 1452 patients who underwent clinical exome sequencing in our laboratory between April 2016 and October 2021. We excluded from this cohort 307 patients who underwent targeted proband-only sequencing, focusing rather on cases where agnostic duo- or trio-sequencing was performed. Detailed phenotyping of the remaining 1145 patients enabled us to define a cohort of 868 patients with NDDs (defined as the presence of at least one of the following: autism spectrum disorder, intellectual disability, developmental delay, learning disability, epilepsy and cerebral palsy, or hypotonia) (Fig. 1A). With this approach we opted for a broad definition of NDDs as diseases affecting the development of the central nervous system and causing functional impairments, and decided to also include patients with isolated epilepsy.

This study was approved by the institutional ethics committee of the Hôpital Universitaire de Bruxelles – Erasme (2021/581) and Hôpital Universitaire de Bruxelles – Hôpital Universitaire des Enfants Reine Fabiola (96/21).

2.2. Mendeliome panel sequencing

Based on OMIM disease genes and an extensive literature review of epilepsy or developmental delay genes, we created a list of 3638 genes associated with Mendelian disorders, as well as overlapping sub-lists of genes specifically associated with epilepsy and with developmental delay. We regularly updated the Mendelian-gene list when new disease-gene associations were described in the literature or through OMIM (4 versions between 2016 and 2021 (Supplementary Table 1)). In its latest iteration the mendeliome panel contained 4867 genes, 1600 of which are associated with NDD. We used these manually curated gene lists to design multiple versions of SeqCap EZ Choice XL Probes (Roche NimbleGen Inc) targeting the coding exons of the selected genes. Library preparation, sequencing and bioinformatic analysis was performed as previously described by Marangoni et al., 2022).

The mean coverage for index samples was between $250 \times$ and $300 \times$, and the parental samples achieved a mean coverage of $150 \times$. In its most recent iteration, 94 % (4561/4867) of the included genes had coverage of $>30 \times$ for at least 95 % of the gene, while 79 % (3824/4867) were covered in their entirety at $>30 \times$. Analysis of intronic regions was limited to -14 and $+6$ base pairs from the exon-intron border, where coverage was $>30x$, permitting confident variant calling.

VCF files were imported into Highlander (<https://sites.uclouvain.be/highlander/>), a bioinformatic tool used for filtering, annotation and visualization of identified variants. We performed trio analysis when DNA from both parents was available. In this case we filtered variants according to mendelian inheritance pattern (de novo, homozygous, compound heterozygous or X-linked), frequency in the population (<0.02 for homozygous and compound heterozygous and <0.01 for de novo and X-linked) and predicted impact on the protein. For patients with only one available parent, “de novo” analysis was limited to NDD or epilepsy genes according to the patient’s phenotype. To identify variants inherited from a parent we furthermore performed a candidate gene analysis (NDD, and Epilepsy gene lists respectively) for patients with an affected parent, and patients with epilepsy. For all patients we also investigated all loss-of-function variants (such as stop, frame-shift or canonical splice-site variants), regardless of whether they were inherited or occurred de novo. Parental imprinting was not taken into account for variant filtering but was assessed on a case-by-case basis for variant interpretation if variants in known imprinted genes were identified.

Incidental findings were reported according to informed consent if clinically actionable. Variants were classified following ACMG guidelines (Richards et al., 2015).

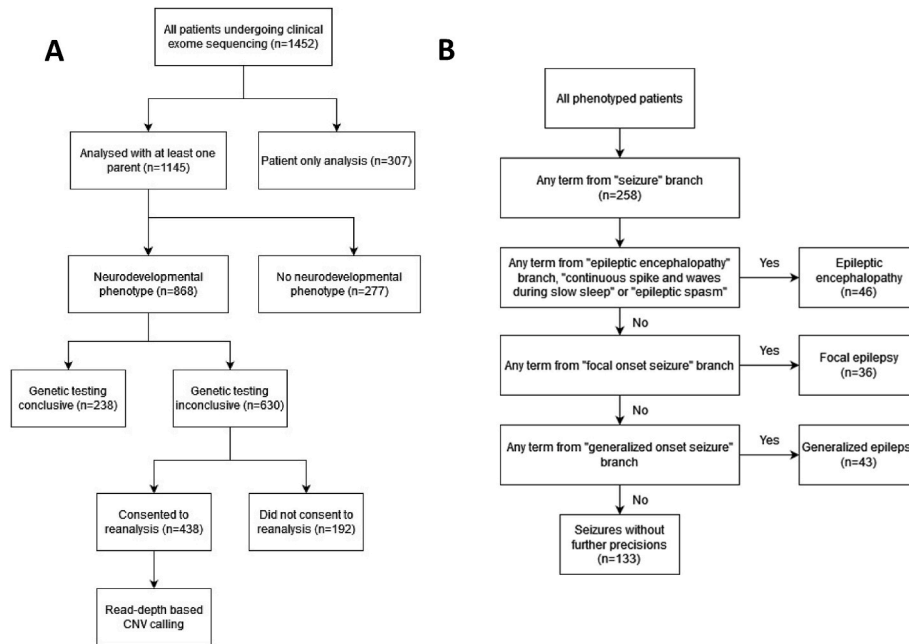


Fig. 1. A. Flowchart illustrating cohort selection, B. Flowchart illustrating stratification of epilepsy branch.

2.3. Phenotyping

Phenotyping was performed based on a manual review of the patients' electronic health record. When such records were unavailable, phenotyping was based on information provided by the prescribing physician on the analysis request form, both in the form of a symptom checklist and free-form commentaries. Diagnosis of ID and ASD was based on results of neuropsychological testing when available, or on the referring physician's appreciation if no objective tests were available. Phenotyping was performed retrospectively at the time of this study, also taking into consideration symptoms that might have arisen after genetic testing. Patients' phenotypes were transcribed into terms of the human phenotype ontology (HPO), searched through the HPO web-interface (<https://hpo.jax.org/app/>). Whenever possible the most specific HPO term for a given symptom was chosen and we aimed to cover all important phenotype abnormalities of the patients, basing our methodology on clinical data encoding guidelines for computational differential diagnosis (Köhler et al., 2019).

2.4. Cohort stratification

Phenotype-based sub-groups were created using HPO terms. Lists of all terms from all main branches of the HPO tree structure, as well as for pertinent sub-branches were extracted from the Ontology-files accessed through <https://hpo.jax.org/app/download/ontology> (release 2021-10-10) using the R package ontologyIndex. Patients were considered to be part of a given sub-group if their phenotype contained at least one term from the given HPO branch. Such defined sub-groups are thus not necessarily mutually exclusive, and a patient can be part of multiple different phenotype categories. A full list of the observed HPO terms per main branch and their prevalence in our cohort can be found in [Supplementary Table 2](#).

HPO-based phenotype categories were defined as: "Intellectual disability" by the presence of at least one term from the intellectual disability HPO branch. "Developmental delay without ID" by the presence of at least one term from the "Neurodevelopmental delay" branch (encompassing motor delay, language delay, delayed social development or global developmental delay) but no ID related terms. A similar process was applied to epilepsy to create the groups "Epileptic encephalopathy", "Focal epilepsy" and "Generalized epilepsy" as illustrated in

Fig. 1B. We defined "ASD" as at least one term from the "Autistic behaviour" branch. This was further stratified into isolated ASD (with only symptoms from the « autistic behavior » or « language delay » HPO branches, ASD with other delay (ID, GDD, or motor delay) and ASD with neither ID/GDD nor motor delay but showing other additional symptoms, such as dysmorphic features or symptoms affecting other organ systems.

The stratification of patients into clinical categories using only HPO branches was complicated by the presence of certain HPO terms in multiple branches (e.g. micro- and macrocephaly being found in the branches "Abnormality of the musculoskeletal system", "Abnormality of head or neck" and "Abnormality of the nervous system"), and branches often containing minor dysmorphic signs, major malformations, and functional symptoms at the same time (such as microcephaly, prominent fingertip pads and hypotonia being all found in the "musculoskeletal abnormality" branch). In addition to categories based on HPO branches we thus manually reviewed all HPO terms observed in our cohort and created three additional categories of "minor dysmorphic features", "major malformation" and "abnormality of neurological examination" ([Supplementary Table 2](#)). We considered morphological anomalies causing significant consequence on health or appearance as major, and anomalies with minimal or no health consequences but modest impact on appearance as minor. We considered patients to have a syndromic phenotype if they presented at least two major or three minor abnormalities.

2.5. Statistical analysis

Continuous variables were described using medians and means, while categorical variables were presented as counts and percentages. For categorical variables, we used Fisher's exact tests for all comparisons between two mutually exclusive sub-groups. Because phenotype categories were not all necessarily mutually exclusive, we compared patients presenting symptoms of a given branch with patients without, rather than patients from one phenotype category against another (e.g., all patients with epilepsy against all patients without epilepsy rather than patients with epilepsy against patients with ID). If more than two sub-groups were compared, we first performed Fisher's exact tests, followed by pairwise Fisher's exact tests if the global test was statistically significant. P-values were corrected using a Benjamini-Krieger-Yekutieli

correction with a false discovery rate of 1 %. Adjusted p-values were considered significant if < 0.05 .

Logistic regression was used to investigate the link between the number of HPO terms per patient and a positive clinical exome result. Mann-Whitney tests were used to compare the number of HPO terms and dysmorphic features in patients for whom a genetic diagnosis could be made against patients for whom genetic testing remained inconclusive. All statistical tests were performed using GraphPad Prism 9.

2.6. CNV-calling

For 438 of the 630 patients for whom mendeliome sequencing was inconclusive and who had given their consent for the reanalysis of their genetic data, we performed CNV calling using the CoNVaDING algorithm (Johansson et al., 2016). Results were visualized through an in-house developed tool (Convader) that produces easily readable graphs of z and ratio-scores and makes it possible to visualize the inheritance of a given variant through a comparison with the parents' samples. CNVs from the CoNVaDING short-list were then manually reviewed considering the observed mode of inheritance, correlation with the patients' phenotype as well as the variant type and size. Candidate CNVs that showed a good clinical correlation were validated through Oxford-nanopore technologies (ONT) long-read sequencing, aCGH 180k or multiplex ligation-dependent probe amplification (depending on the CNV type, size and affected genes) whenever possible. Long-read sequencing was performed either on a PromethION P24 or MinION sequencer with adaptive sampling to target regions of interest and R10.4.1 chemistry. Reads were aligned using minimap2 (version 2.24) and CNVs were called using Sniffles (version 2.0.7).

3. Results

3.1. Cohort characteristics

Between 2016 and 2021 we analyzed 868 duo or trio mendeliome

panels in patients with NDD. Of these patients 531 were male and 337 were female. The mean age at time of analysis was 7.6 years with a median of 5.5 years.

Phenotypes were described by an average number of 6.5 HPO terms and a median of 6 per patient (range from 1 to 36 terms) (Fig. 2A). 1116 different HPO terms were used, the most frequently used being "Delayed speech and language development", "Global developmental delay" and "Autistic behavior" (Supplementary Table 2).

Of the 868 patients, 247 (28 %) presented with ID. The mean age for patients with ID was 11.9 years at time of analysis (median 10.6). Developmental delay without associated ID was noted in 429 (49 %). Of these, 270 had global developmental delay (GDD) or combined language and motor delay. These patients had a mean age of 4.8 years (median 3.6). 31 had isolated motor delay (meaning motor delay with neither language delay nor ID, but potentially associated with other symptoms), and 115 isolated language delay (meaning language delay with neither motor delay nor ID but potentially other symptoms). Mean ages for these patients were respectively 5.2 and 6.7 years (median 2.1 and 4.9 years) (Fig. 3.). The remaining 14 patients were described as "neuro-developmental delay" without further precision.

Cerebral palsy was present in 56 patients (6.5 %). 11 of them presented concomitant ID and 9 presented GDD without ID.

Seizures were described in 258 patients (30 %), epileptic encephalopathy in 46 patients, focal onset epileptic seizures in 36 and generalized onset seizures in 43. For 133 patients the seizure type was not clearly described. 165 patients presented epilepsy with concomitant neurodevelopmental delay. Of these, 63 had ID and 62 had GDD or combined motor and language delay without ID.

Autistic behavior was observed in 192 patients (22 %), of whom 64 had concomitant ID, while 128 did not. Isolated ASD was observed in 19 patients, ASD with other delay in 137 patients and ASD with neither ID/GDD nor motor delay but showing other additional symptoms in 36 patients (Table 1).

After "Abnormalities of the nervous system" (such as developmental delay, epilepsy, ASD, or morphological brain abnormalities), the most

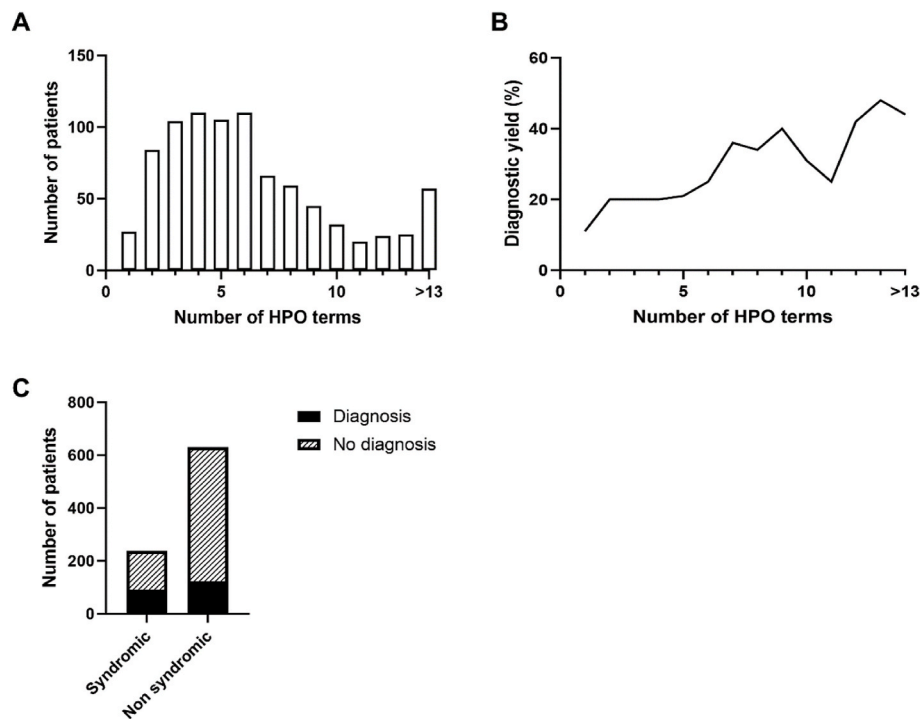


Fig. 2. A. Number of HPO terms per patient. B. Mean diagnostic yield depending on number of HPO terms showing higher yields with increasing numbers of HPO terms. C. Comparison between diagnostic rates in syndromic (43 %, 92/215) and non-syndromic (22 %, 146/653) patients showing higher yields (Fisher's exact p-value < 0.0001) in syndromic presentations.

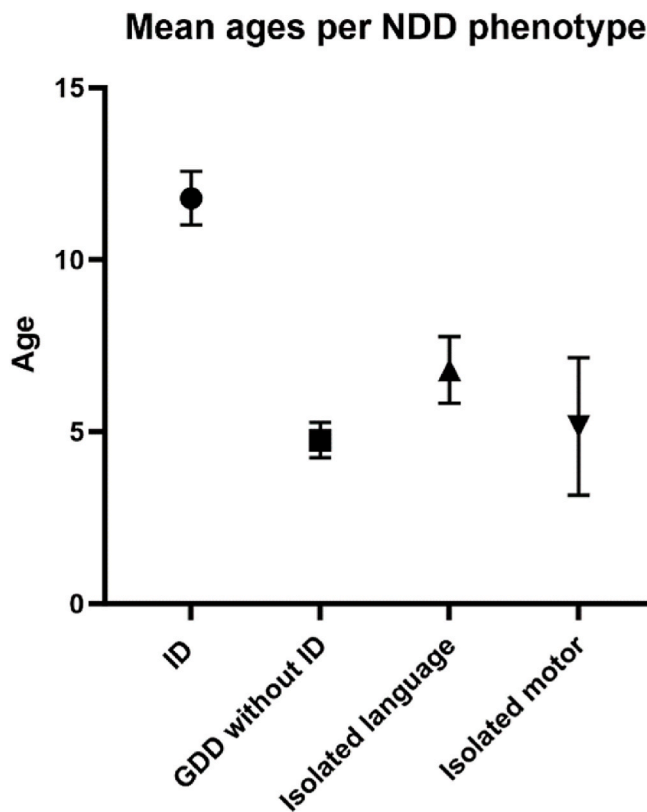


Fig. 3. Mean ages and 95 % CI of patient ages at the moment of genetic testing for different developmental delay categories.

frequently observed phenotypes belonged to the branches “Abnormality of the musculoskeletal system”, in 451 patients, “Abnormalities of head or neck” in 360/846, “Growth abnormalities” in 195, “Abnormality of limbs” in 166 and “Abnormality of the eye” in 162 (Table 2). A detailed description of frequently observed phenotypes and phenotype sub-branches is provided in **Supplementary Results 1**.

We observed “minor dysmorphic features”, such as abnormal facial shape, joint laxity or hypertelorism, in 354 patients, “major malformations” (e.g. scoliosis, cryptorchidism or atrial septal defect (the term major being used here in opposition to minor dysmorphic features)) in 188, and “abnormalities at neurological examination” (e.g. hypotonia, ataxia or spasticity) in 284. A syndromic phenotype (defined as at least

two major or three minor abnormalities) was present in 215 patients.

3.2. Diagnostic yield depending on patients' phenotypes

The overall diagnostic yield was 27 %, with a conclusive genetic diagnosis being reached for 238 patients (Supplementary Table 3). In 9 patients we identified heterozygous pathogenic or likely pathogenic variants in genes for autosomal recessive conditions, with a high phenotypic correlation, but with neither a second variant nor CNV. These cases were thus considered as unresolved. Only variants of unknown significance were reported for 118 patients. 32 incidental findings were reported. Yields were significantly higher in girls (36,8 %, 124/337) than in boys (21,5 %, 114/531) ($p < 0,0001$) (Table 3).

Convincing pathogenic or likely pathogenic variants were identified in 175 different genes. *ITPR1*, *SCN1A* and *PTPN11* genes were each reported in 5 patients and *ANKRD11*, *MECP2* and *NSD1* in 4. Another eight genes were recurrently reported in 3 patients, 29 genes in two, and 132 genes were reported only in a single patient (Supplementary Table 4). All diagnostic variants were submitted to ClinVar (submission SUB15296708).

Of the 238 identified diagnostic variants, the vast majority followed autosomal dominant inheritance (157 cases). Of those, 118 occurred de novo (75 %). Inheritance according to gender is described in detail in Table 4. Lastly, in two patients we observed co-occurrence of pathogenic variants in two different genes following different inheritance patterns (Table 4).

In an 8-year-old girl presenting with developmental delay, cerebellar atrophy, retinitis pigmentosa and dysmorphic features we identified a de novo heterozygous c.4961C > A, p.(Thr1654Lys) variant in *ITPR1*. This variant was considered as likely pathogenic and explained the developmental delay and neurological features but not the retinitis pigmentosa. We also identified compound heterozygous c.3149G > A, p.(Gly1050Asp) and c.1928T > G, p.(Val643Gly) variants in *ABCA4*, which in turn are associated with retinal dystrophy and Stargardt disease and explain the ophthalmological symptoms of the patient.

In a 3-year-old girl presenting with global developmental delay, behavioral problems and dysmorphic features we identified a de-novo occurring c.2278dup p.(Glu760fs) variant in *ADNP*, as well as a maternally inherited c.1529A > G, p.(Gln510Arg) variant in *PTPN11*. Both were considered as likely pathogenic, establishing thus a dual diagnosis of Helsmoortel-Van der Aa and Noonan syndrome.

Mendeliome testing was conclusive in 85 of 247 (34 %) patients with ID. This was significantly higher (p -value = 0.0082) than for patients without ID (25 %, 153/621). A diagnosis was found in 27 % (74/270) of patients with GDD but no ID, 32 % (10/31) for isolated motor and 14 %

Table 1
Diagnostic yields associated with neurological HPO-terms.

Phenotype (Main HPO branches)	Number of patients with phenotype	Number of patients without phenotype	Diagnostic yield for patients with phenotype	Diagnostic yield for patients without phenotype	p-value
ID	247	621	34 %	25 %	0,0083 (**)
Developmental delay without ID	429	439	24 %	31 %	0,0301(*)
GDD/Language and Motor	270		27 %		0,0069 (**)
Isolated motor	31		32 %		
Isolated language	115		14 %		
Seizures	258	610	29 %	27 %	0,5271
Seizures + ID	63		41 %		0,0158(*)
Seizures - ID	195		25 %		
ASD	192	676	16 %	32 %	0,0002 (***)
ASD + ID	64		23 %		0,1103
ASD-ID	128		13 %		
Cerebral palsy	56	812	14 %	28 %	0,0328(*)

For each main category Fisher's exact tests comparing diagnostic yields in patients with the phenotype against patients without the phenotype were performed. For mutually exclusive sub-categories with more than 2 options, p-values refer to global Fisher's exact tests. **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p < 0.05$.

Table 2

Diagnostic yields associated with non-neurological HPO-terms.

Phenotype (Main HPO branches)	Number of patients with phenotype	Number of patients without phenotype	Diagnostic yield for patients with phenotype	Diagnostic yield for patients without phenotype	p-value
Musculoskeletal system	451	417	33 %	21 %	0,0003(****)
Head or neck	360	508	35 %	22 %	0,0001(****)
Growth	195	673	32 %	26 %	0,1542
Limbs	166	702	42 %	24 %	0,0001(****)
Eye	162	706	43 %	24 %	<0,0001(****)
Integument	145	723	40 %	25 %	0,0020(**)
Ear	107	761	44 %	25 %	0,0004(****)
Digestive system	84	784	29 %	27 %	0,6198
Cardiovascular system	72	796	36 %	27 %	0,1284
Genitourinary system	71	797	27 %	27 %	0,6809
Endocrine system	47	821	32 %	27 %	0,4634
Metabolism/homeostasis	41	827	29 %	27 %	0,6579
Immune system	35	833	34 %	27 %	0,3395
Respiratory system	33	835	30 %	27 %	0,5836
Prenatal development or birth	32	836	19 %	28 %	0,3254
Breast	23	845	48 %	27 %	0,0479(*)
Blood and blood-forming tissues	21	847	29 %	27 %	0,6809
Constitutional symptom	15	853	20 %	28 %	0,6067
Neoplasm	12	856	17 %	28 %	0,4706
Voice	3	865	33 %	27 %	0,6809
Cellular phenotype	2	866	50 %	27 %	0,4483

Fisher's exact tests comparing diagnostic yields in patients with the phenotype against patients without the phenotype. ****p ≤ 0.0001, ***p ≤ 0.001, **p ≤ 0.01, *p < 0.05.

Table 3

Differences in diagnostic yield, incidental findings and reported VUS in male and female patients.

	Total (n = 868)	Males (n = 531)	Females (n = 337)	p-value
Confident diagnosis	238 (27 %)	114 (21 %)	124 (37 %)	<0.0001(****)
Incidental finding	32 (3.7 %)	19 (3.6 %)	13 (3.9 %)	0,8547
Heterozygous 4 or 5 variant for AR condition	9 (1.0 %)	7 (1.3 %)	2 (0.6 %)	0,4947
VUS	118 (14 %)	91 (17 %)	27 (8.0 %)	0,0001(****)
No diagnostic variant reported	471 (54 %)	300 (56 %)	171 (51 %)	0,1079

Fisher exact tests between males and females for a given condition against males and females without the given condition. ***p ≤ 0.001, **p ≤ 0.01, *p < 0.05, ****p ≤ 0.0001.

(16/114) for language delays (Table 1). When comparing ID, GDD without ID and isolated motor delay, diagnostic yields did not differ significantly, however all three showed significantly higher yields than patients with isolated language delay.

A confident diagnosis was reached for 74 of the 258 patients suffering from epilepsy (29 %). Yield was 24 % (11/46) for patients with epileptic encephalopathy, 22 % (8/36) for patients with focal epilepsy, 19 % (8/43) for patients described as generalized onset epilepsy and 35 % (47/133) for the remaining patients with epilepsy without further specification. For patients with epilepsy and concomitant ID, diagnostic yields were significantly higher than for patients with epilepsy without ID (41 % (26/63) vs 25 % (48/195), p-value = 0,0158) (Table 1). When looking at patients with epilepsy and either ID or GDD vs neither ID nor GDD these differences were less striking (36 % (45/125) vs 22 % (29/133)) but remained statistically significant (p-value = 0.0133).

Diagnostic yield was especially low in ASD patients, reaching 16 % (31/192) for patients with ASD against 31 % (207/676) for patients

Table 4

Differences in heredity of variants reported as confident diagnosis in male and female patients.

	Total (n = 238)	Males (n = 114)	Females (n = 124)	Significance
Autosomal dominant	157 (66 %)	83 (73 %)	74 (60 %)	0,0400(*)
AD - de novo	118 (50 %)	63 (55 %)	55 (44 %)	0,1193
AD - inherited	17 (7.1 %)	10 (8.8 %)	7 (5.6 %)	0,4517
AD - unknown	22 (9.2 %)	10 (8.8 %)	12 (10 %)	0,8272
Autosomal recessive	54 (23 %)	20 (18 %)	34 (27 %)	0,0881
AR - homozygous	35 (15 %)	13 (11 %)	22 (18 %)	0,2010
AR - compound heterozygous	19 (8.0 %)	7 (6.1 %)	12 (10 %)	0,3478
X-linked	25 (11 %)	11 (10 %)	14 (11 %)	0,8329
XL - inherited	7 (2.9 %)	7 (6.1 %)	0 (0 %)	0,0046(**)
XL de novo	15 (6.3 %)	3 (2.6 %)	12 (10 %)	0,0319(*)
XL unknown	3 (1.3 %)	1 (0.9 %)	2 (1.6 %)	>0,9999
Multiple	2 (0.8 %)	0 (0 %)	2 (1.6 %)	0,4988

Fisher exact tests between males and females for a given condition against males and females without the given condition. ****p ≤ 0.0001, ***p ≤ 0.001, **p ≤ 0.01, *p < 0.05.

without ASD (p-value = 0.0002). For the subgroup of patients presenting with ASD and any other form of developmental delay (ID, GDD or motor delay), a molecular diagnosis was reached in 20 % of cases (27/137). This reached up to 23 % (15/64) when focusing on patients with ASD + ID. In patients with ASD, neither ID, GDD nor motor delay, but with any other additional symptoms (such as dysmorphic features, epilepsy, or symptoms concerning other organs) diagnostic yield was 11 % (4/36). On the contrary, all 19 patients with isolated ASD remained unsolved (0 % (0/19) vs 18 % (31/173) for patients with any additional symptom (p-value = 0,0472) (Table 1). Similarly to what we observed in the whole cohort, yields were significantly higher in girls with ASD (28 %, 14/50) than in boys (12 %, 17/142) (p-value = 0,0130).

Several secondary phenotypes were associated with an increase in diagnostic yield. Most significant differences were seen in patients with

“Eye abnormalities” (44 % vs 24 %), “Abnormality of the musculoskeletal system” (33 % vs 21 %), “Abnormality of head or neck” (35 % vs 22 %) and “Abnormality of limbs” (42 % vs 24 %), all with a p-value <0.0001. Other categories with significantly higher yields were “Ear abnormalities”, “Abnormalities of the integument” and “Abnormality of breast” (Table 2). An in-depth description of phenotypes associated with significantly higher diagnostic yields is provided in **Supplementary Results 2**.

We observe that the presence versus absence of phenotypes considered as “minor dysmorphic features” is associated with a diagnostic yield of 35 % (125/354) vs 22 % (113/514) (p-value < 0.0001), while for “major malformations” it is 36 % (67/188) vs 25 % (171/680) (p-value = 0.0107) and for abnormalities at neural examination 35 % (100/284) vs 24 % (138/584) (p-value = 0.0014). Logarithmic regression showed that the probability of finding a molecular diagnosis increases with the number of associated HPO terms (p-value <0.0001). For patients with only 1 HPO term, diagnostic yield was 11 % (3/27), while in patients with 13 or more HPO terms a confident diagnosis was made in 45 % of cases (37/82) (Fig. 1B). Patients with positive genetic testing also showed significantly more “minor dysmorphic features” (mean of 2.11 vs 1.01, p-value <0.0001) and “major malformations” (mean 0.51 vs 0.33, p-value = 0.0031).

The vast majority of observed terms in the branches “abnormalities of the breast”, “integument”, “head and neck” as well as “abnormalities of the limbs” were also part of our manually curated list of minor and major dysmorphic features (respectively 100 %, 90 %, 88 % and 87 % of observed terms). For the categories “ears”, “eyes” and “musculoskeletal”, the fraction of dysmorphic features was 62 %, 41 % and 54 % respectively, with more terms describing functional anomalies such as “hearing loss” or “hypotonia” rather than dysmorphic features. To assess if differences in diagnostic yield for these HPO branches were mainly due to dysmorphic features we also assessed diagnostic yields after excluding all minor and major dysmorphic terms. Only abnormality of the ears, eyes, limbs, and musculoskeletal system remained significant when excluding dysmorphic features: abnormality of the ears excluding dysmorphic features (e.g. hearing impairment): 48 % (25/52) vs 26 % (213/816), p-value = 0.0026; abnormality of the eyes excluding dysmorphic features (e.g. nystagmus): 41 % (45/111) vs 25 % (193/757), p-value = p.0028; abnormality of the limbs excluding dysmorphic features (e.g. hemihypertrophy or limb hypertonia): 54 % (14/26) vs 27 % (224/842), p-value = 0.0107; musculoskeletal abnormality excluding dysmorphic features (e.g. microcephaly, spasticity): 35 % (123/350) vs 22 % (115/518), p-value = 0.0002.

Diagnostic yield in syndromic patients was significantly higher than in non-syndromic patients (43 % (92/215) vs 22 % (146/653), p-value <0.0001) (Fig. 2C). Due to the high impact of a syndromic presentation in our population we assessed if this effect was present in all three main

NDD phenotypes (ID, ASD, and epilepsy). In syndromic vs non-syndromic cases of ID, diagnostic yields were respectively 57 % (30/53) and 28 % (55/194) (p-value = 0.0003), in ASD 32 % (12/38) and 12 % (19/135) (p-value = 0.0067), and in epilepsy 50 % (19/38) vs 25 % (55/220) (p-value = 0.0031).

3.3. Added diagnostic yield through sequencing based CNV calling

Called CNVs were reviewed considering family segregation, mode of heredity and phenotypic correspondence.

We retained 12 variants as potentially diagnostic for further validation (Table 5) and were able to confirm the presence of seven with a complementary technique. In three cases, the variant was confirmed by aCGH (where either sequencing was performed before aCGH or reanalysis of a previously performed negative aCGH led to the identification of the variant), by MLPA in one case, by Whole Exome sequencing with CNV calling using ExomeDepth (Plagnol et al., 2012) in one case, and by ONT sequencing in two. Both cases confirmed through ONT sequencing concerned intragenic duplications where ONT not only enabled us to confirm the presence of the CNV, but also to confirm that both variants lead to frameshifts causing premature terminating codons. Three patients were not available for additional genetic testing, but the identified variants were still considered as convincing due to their good correlation with the patients’ phenotypes, as well as the convincing aspect of ratio and z scores visualized through Convader. CNV calling using the CoN-VaDING algorithm thus led to ten additional diagnoses in our CNV-calling cohort of 438 patients (2,3 %). A short clinical description of all confirmed cases is provided in **Supplementary Results 3**. In two cases variants could not be confirmed through ONT and were thus considered as false positive calls.

4. Discussion

Whole exome sequencing (WES) has profoundly changed the diagnostic approach for children affected by rare NDD. At the start of the study, WES presented some caveats such as non-uniform coverage, with sub-optimal variant calling in low-coverage regions (Wang et al., 2017). Similar issues have been observed with clinical exome panels where coverage and performance has been shown to be heterogenous between different capture kits (Pengelly et al., 2020). To address these issues, we have chosen to manually curate a list of disease-causing genes and to perform deep sequencing at ~300 × using in-house designed SeqCap EZ Choice XL Probes. This allowed us to cover all genes of interest, while maintaining good coverage of all targeted regions, at a reasonable cost.

The overall diagnostic yield was 27 % in our cohort of patients with NDDs, which is in line with the diagnostic yield of WES for NDDs reported at 27.2 % in a recent meta-analysis (Stefanski et al., 2021)

Table 5
Variants of interest identified through CNV calling in previously undiagnosed patients.

Patient	Variant	Size	Targets	Gene	Heredity	Validation	Diagnosis
81	chr7:5567367-5569299 DEL	1932	5	<i>ACTB</i>	AD, de novo	aCGH 180k	+
87	chrX:153577205-153792687 DUP	215482	107	<i>RPL10</i>	XL	aCGH 180k	+
182	chr4:107092217-107092476 DEL	259	1	<i>TBCK</i>	AR, compound heterozygous	ONT positive	+
	chr4:107114708-107151647 DUP	36939	20				
215	chr4:25972452-25973296 DUP	844	4	<i>ASXL2</i>	AD, de novo	ONT positive	+
243	chr6:79649561-79657551 DEL	7990	8	<i>PHIP</i>	AD, de novo	WES + ExomeDepth	+
310	chr19:917447-920689 DEL	3242	9	<i>KISS1R</i>	AR, homozygous	MLPA	partial
597	chr12:23998866-23999177 DEL	311	1	<i>SOX5</i>	AD, de novo	aCGH 180k	+
22	chrX:7137672-7268313 DEL	130641	10	<i>STS</i>	XL inherited	Not possible	?
57	chr22:50665150-50665517 DEL	367	2	<i>TUBGCP3</i>	AR: compound heterozygous with SNV	Not possible	?
97	chr15:75676593-75676789 DUP	196	1	<i>SIN3A</i>	AD, de novo	Not possible	?
134	chr6:8420249-8421611 DUP	1362	6	<i>RERE</i>	AD, de novo	ONT negative	-
765	chr9:130442385-130442571 DUP	186	1	<i>STXBPI</i>	AD, de novo	ONT negative	-

Coordinates, size and number of significantly altered targets are derived from the CoNVaDING output. In case of CNVs comprising multiple genes only the main gene expected to cause the patient’s phenotype is reported in the Gene column.

ONT: Oxford Nanopore technologies long-read sequencing, aCGH: array comparative genome hybridization, MLPA: multiple ligation probe assay.

investigating yields of WES and gene-panels. This suggests that well-designed clinical exome sequencing might yield results similar to WES at lower sequencing and data-handling costs but at the expense of reusability in clinics and in research. With diminishing sequencing costs, more efficient recapture kits and possibility of reanalysis, WES has become the clinical sequencing first-line test in NDDs (Srivastava et al., 2019), leaving high sequencing depth panels and clinical exome a possible niche in the search of low-grade mosaicism (Stosser et al., 2018), and scenarios where 100 % coverage of a region is required.

ID/GDD was associated with significantly higher diagnostic yields when comparing patients with and without the given phenotype. GDD and ID are closely related, with GDD being defined as a significant delay in two or more developmental domains in a child aged 5 years or younger, while the term ID is generally reserved for patients older than 5 years. Because most patients with GDD will meet the diagnostic criteria for ID when assessed later in life, it has been suggested that both terms largely describe the same condition in different age groups (Bélanger and Caron, 2018). Accordingly, the mean age of ID patients was 11.9 years against 4.8 years for GDD patients in our cohort. When grouping together ID and GDD patients we observed an overall diagnostic yield of 31 % (358/517) for ID/GDD, with yields slightly but not significantly higher in ID patients (34 %) than in patients with GDD but no ID (27 %). Diagnostic yield when only language development was delayed was lower at only 14 %. This low yield in patients with isolated language delay might be partially due to an overlap with ASD patients or be due to the fact that language delay with neither ID nor motor delay is a milder phenotype which might be more likely to be due to oligogenic factors.

ASD, especially when isolated (i.e. without ID, delayed motor milestones or any other associated symptoms), was associated with a significantly lower diagnostic yield. ASD is a phenotypically heterogeneous condition, with a complex genetic architecture, comprised of a combination of de novo or inherited high, moderate, and low impact variants, de novo high impact variants being associated with more complex and severe phenotypes (Antaki et al., 2022). Cases with high autism polygenic scores, linked to often inherited moderate or low impact variants, on the other hand have been associated with a lower likelihood of co-occurring developmental disabilities (Warrier et al., 2022). This might explain why a causal variant is more frequently identified in more severe cases, while isolated forms often remain unresolved (Tammimies et al., 2015). These polygenic forms might partially explain the particularly low diagnostic yield in ASD compared with other NDDs (Stefanski et al., 2021).

The presence of epilepsy related symptoms on the other hand did not have a significant impact on diagnostic yields compared to patients without epilepsy. While the presence of ID in patients with epilepsy increases the rate of molecular diagnosis, epilepsy types had no impact on diagnostic yields, even though higher yields have been reported for generalized epilepsy compared to focal forms in the literature (Stefanski et al., 2021). Genetic causes of epilepsy show a high phenotypic heterogeneity with a single gene or even a single variant often causing very different epilepsy phenotypes in different patients. This variability might be explained by a complex interplay between rare high-impact variants which generally occur de novo and are classically associated with severe phenotypes of epileptic encephalopathy, and common variants, classically associated with common epilepsies in an oligogenic or polygenic fashion (Ellis et al., 2020).

Additional factors that were highly predictive of identifying a genetic diagnosis were female gender, as well as the presence of more complex and syndromic phenotypes and dysmorphic features. Males were over-represented in our cohort with a female to male ratio of 1:1.58, close to the 1:1.7 ratio described in the literature (Bölte et al., 2023). In ASD patients this difference was especially high at 1:2.8 which is in line with the known higher sex discrepancy, estimated at about 1:3–4 in the literature (Bölte et al., 2023). Diagnostic yield however was significantly higher in girls both in our overall and ASD cohort. Similar differences in diagnostic yield have previously been observed in other studies (Gieldon

et al., 2018). This gender discrepancy has been suspected to be due to a female protective model in NDD, according to which the female brain might be protected against certain NDD, and particularly against ASD (Jacquemont et al., 2014). A proposed model is that boys and girls might present different disease thresholds. In that model, the occurrence of NDD in girls would require the presence of more, and/or more severe variants, such as high impact de novo variants. On the contrary in boys, the presence of a combination of lower impact inherited variants may be sufficient for the phenotype to occur (Antaki et al., 2022; Jacquemont et al., 2014). It should however be noted that there is no clear molecular explanation for this female protective effect, and some recent studies have pointed out shortcomings of the predominant “differing thresholds under a liability threshold” model (Dougherty et al., 2022).

The higher the number of HPO terms used to describe a patient’s phenotype, the higher the diagnostic yield, ranging from about 10 % in patients with a single HPO term to approximately 45 % for patients with 13 or more HPO terms. This is in line with results in the literature where yields were higher in syndromic ID/NDD (Martinez-Granero et al., 2021). When analyzing yields associated with all main HPO branches individually, anomalies of the ears, eyes, limbs, integuments, skeletal system, head and neck, and breast were all associated with significantly higher yields in patients presenting the phenotype versus patients without the phenotype (Table 2). Dysmorphic features made up the vast majority of terms observed in abnormalities of the integument, head and neck and breast. The presence of minor dysmorphic features was associated with significantly higher yields, and patients with a genetic diagnosis showed more minor dysmorphic signs than those with inconclusive genetic testing. The same was true for a manually curated list of major malformations. Similar observations have been made in the past concerning aCGH analysis and WES, linking dysmorphic features and congenital anomalies to a higher probability of finding a pathogenic variant (Martinez-Granero et al., 2021). Branches which remained significantly associated with higher diagnostic yields even after removing dysmorphic terms were abnormalities of the ears, eyes, musculo-skeletal system (containing numerous terms related to hypotonia) and of the limbs (where mostly terms relating to limb hypertonia remained). Those observations highlight the importance of dysmorphic features, phenotypic complexity, and syndromic presentations as predictive factors of a monogenic disorder.

Our results suggest that NGS obtains very high yields for certain phenotypes, notably ID. Yields for ASD patients were generally low, but higher yields were achieved in sub-groups where a monogenic inheritance and high-impact variants are expected, such as in girls and patients with more complex phenotypes. The benefits of genetic testing in patients with isolated ASD, which will more often present a polygenic inheritance on the other hand, are less clear.

It should however be noted that a recent study on 299 toddlers with ASD who underwent genetic testing yielded contradictory conclusions, and stated a lack of phenotypic features differentiating patients with or without pathogenic variants (Harris et al., 2020). Further research is necessary to assess if clinical features can be reliably used to select patients with suspected monogenic forms of ASD for genetic testing.

CNV calling on NGS data led to an additional diagnostic yield of about 2 % in our cohort, where most patients had a previous negative aCGH. This observation is in line with data from the literature suggesting yields between 1 and 2 % (Marchuk et al., 2018; Testard et al., 2022). In a population with no preceding aCGH testing this yield is expected to be higher and has been shown to reach up to 19 % (Zhai et al., 2021).

The clinical interpretation of identified CNVs is challenging, especially in the case of intragenic duplications which can be either deleterious or clinically insignificant depending on whether the insertion disrupts an open reading frame. Oxford-nanopore long read sequencing has been used in the past to help in the clinical interpretation of intragenic duplication (Bai et al., 2022). For 2 patients in our cohort, we were able to interpret the observed variants through a detailed

characterization of CNV breakpoints using ONT-LRs. These results highlight the benefits of NGS based CNV calling, even when aCGH is negative, and present ONT-LRs as an efficient way to validate identified CNVs.

In conclusion, we show that clinical exome sequencing with additional read-depth based CNV-calling can reach yields similar to those seen with WES or WGS in patients with neurodevelopmental disorders. A detailed analysis of patient phenotypes revealed ID/GDD to be an important predictor for finding a molecular diagnosis. Additional factors associated with significantly higher yields were female gender and the presence of minor dysmorphic features or syndromic phenotypes. The high diagnostic yield observed further underscores the importance of systematically performing NGS-based genetic testing in these patient groups.

CRediT authorship contribution statement

Sebastian Neuens: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Julie Soblet:** Writing – review & editing, Resources, Investigation. **Aurelie Penninckx:** Writing – review & editing, Investigation, Data curation. **Claire Detry:** Writing – review & editing, Investigation. **Cindy Badoer:** Writing – review & editing, Investigation. **Laurence Desmyter:** Writing – review & editing, Investigation. **Xavier Peyrassol:** Writing – review & editing, Investigation. **Françoise Wilkin:** Writing – review & editing, Investigation. **Adeline Busson:** Writing – review & editing, Investigation. **Marie Bruneau:** Writing – review & editing, Investigation. **Marie-Laure Grenet:** Writing – review & editing, Investigation. **Alice Le Morillon:** Writing – review & editing, Investigation. **Alec Aeby:** Writing – review & editing, Investigation. **Nicolas Deconinck:** Writing – review & editing, Investigation. **Cynthia Prigogine:** Writing – review & editing, Investigation. **Anne Monier:** Writing – review & editing, Investigation. **Elodie Juvené:** Writing – review & editing, Investigation. **Tom Balfroid:** Writing – review & editing, Investigation. **Audrey Van Hecke:** Writing – review & editing, Investigation. **Florence Christiaens:** Writing – review & editing, Investigation. **Chantal Depondt:** Writing – review & editing, Investigation. **Cécile Brachet:** Writing – review & editing, Investigation. **Veronique Delvenne:** Writing – review & editing, Investigation. **Nicolas Lufin:** Writing – review & editing, Software, Resources. **Youssef Bouysran:** Writing – review & editing, Software, Resources. **Molka Kammoun:** Writing – review & editing, Investigation, Data curation. **Dorien Daneels:** Writing – review & editing, Resources. **Ben Caljon:** Writing – review & editing, Resources. **Didier Croes:** Writing – review & editing, Resources. **Catharina Olsen:** Writing – review & editing, Resources. **Sonia Van Dooren:** Writing – review & editing, Resources. **Isabelle Migeotte:** Writing – review & editing, Investigation. **Isabelle Vander-noot:** Writing – review & editing, Investigation. **Martina Marangoni:** Writing – review & editing, Resources, Methodology, Investigation. **Sandra Coppens:** Writing – review & editing, Investigation. **Guillaume Smits:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Catheline Vilain:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmg.2025.105030>.

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Data availability

The data that has been used is confidential.

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