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## ORIGINAL ARTICLE

# Efficient strategy for the molecular diagnosis of intellectual disability using targeted high-throughput sequencing

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► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/jmedgenet-2014-102554>).

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Received 1 June 2014  
Revised 31 July 2014  
Accepted 3 August 2014  
Published Online First  
28 August 2014



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**To cite:** Redin C, Gérard B, Lauer J, et al. *J Med Genet* 2014;**51**:724–736.

## ABSTRACT

**Background** Intellectual disability (ID) is characterised by an extreme genetic heterogeneity. Several hundred genes have been associated to monogenic forms of ID, considerably complicating molecular diagnostics. Trio-exome sequencing was recently proposed as a diagnostic approach, yet remains costly for a general implementation.

**Methods** We report the alternative strategy of targeted high-throughput sequencing of 217 genes in which mutations had been reported in patients with ID or autism as the major clinical concern. We analysed 106 patients with ID of unknown aetiology following array-CGH analysis and other genetic investigations. Ninety per cent of these patients were males, and 75% sporadic cases.

**Results** We identified 26 causative mutations: 16 in X-linked genes (*ATRX*, *CUL4B*, *DMD*, *FMR1*, *HCFC1*, *IL1RAPL1*, *IQSEC2*, *KDM5C*, *MAOA*, *MECP2*, *SLC9A6*, *SLC16A2*, *PHF8*) and 10 de novo in autosomal-dominant genes (*DYRK1A*, *GRIN1*, *MED13L*, *TCF4*, *RAI1*, *SHANK3*, *SLC2A1*, *SYNGAP1*). We also detected four possibly causative mutations (eg, in *NLGN3*) requiring further investigations. We present detailed reasoning for assigning causality for each mutation, and associated patients' clinical information. Some genes were hit more than once in our cohort, suggesting they correspond to more frequent ID-associated conditions (*KDM5C*, *MECP2*, *DYRK1A*, *TCF4*). We highlight some unexpected genotype to phenotype correlations, with causative mutations being identified in genes associated to defined syndromes in patients deviating from the classic phenotype (*DMD*, *TCF4*, *MECP2*). We also bring additional supportive (*HCFC1*, *MED13L*) or unsupportive (*SHROOM4*, *SRPX2*) evidences for the implication of previous candidate genes or mutations in cognitive disorders.

**Conclusions** With a diagnostic yield of 25% targeted sequencing appears relevant as a first intention test for the diagnosis of ID, but importantly will also contribute to a better understanding regarding the specific contribution of the many genes implicated in ID and autism.

## INTRODUCTION

Intellectual disability (ID) is a common neurodevelopmental disorder reported in 1.5–2% of children and adolescents.<sup>1,2</sup> ID is defined by significant limitations in both intellectual functioning and adaptive behaviour with onset before the age of 18. Different classes of ID are conventionally defined upon IQ values (severe or profound, <35; moderate, 35–49 and mild, 50–70). However, in routine genetic practice, clinical assessment mainly based on records of developmental history, speech acquisition and patients' autonomy is used for classification in such subcategories.

Causes of ID can be environmental, genetic or multifactorial. Single genetic events are thought to account for a majority of cases, varying from large chromosomal anomalies or copy number variants (CNVs) affecting several genes to point mutations in single genes. These latter monogenic forms are characterised by an extreme genetic heterogeneity, with a hundred genes described as implicated in X-linked ID (XLID), and more associated to autosomal-recessive or autosomal-dominant forms. Altogether there are more than 500 genes proposed to cause ID with high penetrance when mutated<sup>3–9</sup> underlying a phenotypic heterogeneity of the same extent in both severity and associated symptoms. This genetic heterogeneity has long limited the diagnostic offer for patients and families, which

was often restricted to fragile-X (MIM 300624) testing, array-CGH (comparative genomic hybridization) analysis and generic metabolic tests (see online supplementary figure S1). It may be complemented by sequencing a few genes associated to a specific syndrome evoked by patients' phenotype, yet the diagnostic yield remains low (1–2% for the recurrent fragile-X mutation; 10–15% for array-CGH and chromosomal analyses, higher in highly syndromic patients).<sup>10–12</sup> A majority of patients remain therefore without molecular diagnosis, while it is of crucial importance for establishing recurrence risks and providing genetic counselling in the family. Moreover, such diagnosis often has direct consequences for the medical prognosis of patients or their optimised healthcare, and even (yet in still a minority of cases) can indicate specific therapeutic options.

To obviate this low diagnostic yield, we developed the simultaneous targeted sequencing of protein-coding exons of 217 genes associated with ID or autism spectrum disorders (ASDs) as primary clinically significant feature: 99 located on the X-chromosome, 118 on the autosomes. We report here the results of such strategy on a cohort of 106 ID patients with or without associated autistic-like features, negative for array-CGH, fragile-X and other specific genetic analyses. A causal mutation was detected in 25% of these patients, regardless the severity of their cognitive impairment. We illustrate cases in which the molecular diagnosis was immediately established, as well as other more complex situations. This highlights the challenge of interpreting variants generated by NGS technologies, already from targeted approaches restricted to a few hundred genes. This work demonstrates that a targeted sequencing approach is highly efficient for the diagnosis of ID, but also allows refining the clinical spectrum associated with mutations in certain genes, and confirming or questioning the involvement of other genes in cognitive disorders.

## METHODS

### Cohort of patients

DNA samples from 106 patients were addressed for testing through clinical geneticists from 16 public hospitals in France. Inclusion criteria for the patients were to be negative for the recurrent fragile-X mutation and for pathogenic CNVs via array-CGH testing, and availability of DNA samples from family members in order to conclude upon molecular findings. Additional specific genetic investigations had been performed on a majority of patients (on average: two genes tested per patient, table 1). Presence of multiple major congenital anomalies or suspected mitochondrial/peroxisomal disorders was an exclusion criterion. Clinical data were recorded following a standardised clinical record highlighting prenatal history, developmental history, neurological and behavioural disorders. ID severity was assessed by medical geneticists upon clinical evaluation and was not a discriminating inclusion criterion, although we encouraged inclusion of probands with moderate or severe ID. This study was approved by the local Ethics Committee of the Strasbourg University Hospital (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPRB)). For all patients, a written informed consent for genetic testing was obtained from their legal representative.

### Targeted genes and capture design

The 217 selected genes include 99 genes associated to XLID and 118 genes located on autosomes, implicated in dominant (45), recessive (66) or complex (7) forms of ID (see online supplementary table S8, further justification on gene selection is given in online supplementary methods). We targeted all

protein-coding exons of these genes, including 20 bp of intronic flanking sequences. The overall size of targeted regions is 1.034 Mbp. Corresponding 120 bp RNA baits were designed using SureDesign (<https://array.chem.agilent.com/suredesign/>).

### Library preparation and sequencing

DNA samples were extracted from peripheral blood or saliva. Sequencing libraries were prepared as described previously,<sup>13</sup> performing individual in-solution SureSelect capture reaction for each DNA sample (Agilent, Santa Clara, California, USA). Paired-end sequencing (2×101-bp) was performed on an Illumina HiSeq 2000/2500, multiplexing up to 16 samples per sequencing lane.

### Bioinformatic pipeline and variant ranking

Read mapping, variant calling and annotation was performed as described previously.<sup>13</sup> Detected variants (short indels and single nucleotide variants (SNVs)) were ranked by VaRank (an in-house developed script), which incorporates the annotations retrieved by alamut-HT (putative effect on the protein, conservation scores, splice site predictions, allelic frequency in the 106 patients and in control cohorts such as Exome Variant Server (EVS) or 1000 genomes). Candidate variants were selected when harbouring a frequency compatible with the incidence of the disease: expectedly accounting for less than 0.1% of all ID cases, aka resulting in a disease frequency <0.002%.<sup>14</sup> Using the EVS population as a subset of the general population, candidate variants were thus retrieved when reported in EVS: with a minor allele frequency lower than 0.45% for variants in autosomal-recessive genes (ie, with a frequency of homozygotes <0.002%), in no more than one carrier for variants in autosomal-dominant genes or in no more than one male for variants in X-linked genes (as we cannot exclude, although it is unlikely, that a particular carrier from the general population might have mild ID). We concomitantly excluded variants present more than twice in the cohort of 106 patients. Remaining variants predicted as potentially pathogenic and fitting with the mode of inheritance associated to the affected gene were further tested for validation (see online supplementary table S9).

### CNVs detection pipeline

Putative heterozygous/homozygous/hemizygous structural variants or CNVs were highlighted using the previously described method based on a depth-of-coverage comparison between the index sample and eight other random samples from the same sequencing lane.<sup>13</sup> For the X-chromosome, coverage was normalised according to the number of X-chromosomes of the patient.

### Mutation validation

All candidate mutations were validated by Sanger sequencing and co-segregation analyses were performed as extensively as possible. Putative splicing mutations were confirmed either using a minigene *in vitro* assay with the SPL3B plasmid as described previously<sup>15</sup> or using patients' fibroblasts or blood RNA when available. For apparent *de novo* variants, pedigree concordance was checked using polymorphic microsatellite markers (PowerPlex 16HS System, Promega). Mutations were considered as certainly causative when no doubt remained regarding their pathogenicity and an unambiguous diagnosis could thus be established. Such mutations co-segregated with the disease status in the family and were either truncating mutations or missense mutations that had been previously

**Table 1** Description of the cohort of 106 patients with intellectual disability (ID) and global diagnostic results

	Cohort (n=106)	With conclusive genetic diagnosis (n=26)			Yield (per category)
<b>Gender</b>		XLID	ADID	Total	
Male	96 (91%)	14	8	22	22/96 (23%)
Female	10 (9%)	2	2	4	4/10 (40%)
<i>Total</i>	<i>106</i>	<i>16</i>	<i>10</i>	<i>26</i>	<i>26/106 (25%)</i>
<b>Age</b>					
[0–10]	57 (54%)	9	5	14	14/57 (25%)
[10–20]	31 (29%)	5	4	9	9/31 (29%)
>20	18 (17%)	2	1	3	3/18 (17%)
<b>Sporadic cases *</b>					
Female	8 (7%)	2	2	4	4/8 (50%)
Male	72 (68%)	7	7	14	14/72 (19%)
<i>Total</i>	<i>80 (75%)</i>	<i>9</i>	<i>9</i>	<i>18</i>	<i>18/80 (23%)</i>
<b>Familial history</b>					
Male sib-pairs	12	5	0	5	5/12 (42%)
Possible XLID†	8	2	1	3	3/8 (38%)
Other (non-X-linked)	6	0	0	0	0/6 (0%)
<i>Total</i>	<i>26 (25%)</i>	<i>7</i>	<i>1</i>	<i>8</i>	<i>8/26 (31%)</i>
Consanguinity	3 (3%)	0	0	0	0/3 (0%)
<b>ID severity</b>					
Mild/borderline	12 (11%)	3	0	3	3/12 (25%)
Moderate	49 (46%)	4	6	10	10/49 (20%)
Severe	45 (42%)	9	4	13	13/45 (29%)
<b>Comorbidity</b>					
Microcephaly (<–2 SD)	14 (13%)	3	2	5	5/14 (36%)
Epilepsy	28 (26%)	5	2	6	6/28 (21%)
Autistic traits	34 (32%)	7	2	9	9/34 (26%)
Hypotonia	36 (34%)	4	4	8	8/36 (22%)
<b>Previous exploration</b>					
CGH	106 (100%)				
Fragile-X	105 (99%)				
Karyotype	99 (96%)				
# Other genetic tests (mean per patient)	2				
MRI	53 (50%)				
Metabolism‡	82 (77%)				

\*No familial first degree ID.

†Affected male relatives.

‡At least one biochemical test performed.

XLID, X-linked ID; in *italic*: total number per category.

convincingly published or that we confirmed with functional analyses. Mutations were considered as potentially causative when they appeared to co-segregate with ID in available members of the family and were predicted to be damaging, but further functional studies are needed to prove unambiguously their pathogenicity.

## RESULTS

### High-quality sequencing data ensure low rates of false-positive/negative calls for SNVs, indels and CNVs

Our strategy allowed generating a high-quality sequencing dataset, with a mean depth of coverage of 350× and an average per patient of 97.7% of targeted regions being well covered (>40×; see online supplementary table S1). Such coverage ensures a sensitivity of 99–99.9% of detecting SNVs and indels at any allelic state (Illumina Technical Note). We further assessed the sensitivity of SNV detection by comparing allelic states of SNPs detected by SNP-array (Affymetrix SNP Array 6.0) with the corresponding sites located in the targeted sequencing data of two patients and found no false-negatives after Sanger

sequencing validation of six SNPs showing discrepant allelic states between both methods (452 SNPs analysed). Interestingly, for these six SNPs, Sanger sequencing results were always in favour of targeted sequencing data suggesting a much higher accuracy (data not shown). No false positive was detected out of the 80 candidate variants located in well-covered regions that were tested for confirmation by Sanger sequencing.

This high-sequencing depth also ensured reliable CNVs calling. All CNVs detected by our pipeline were validated by Sanger sequencing, qPCR and/or confirmed retrospectively when looking at array-CGH data (see online supplementary table S2). Some were not initially mentioned in the array-CGH report because they were covered by only a few SNP probes (≤2 deleted probes, under the detection threshold).

Very few regions (a total of 3.9 kb, only 1.8 kb being protein coding) appear consistently poorly covered (coverage <40× in >90% of the samples; see online supplementary table S3). Those are mainly first exons or highly GC-rich regions that are a well-known burden in such capture strategy.

### Cohort description and diagnostic yield

Patients harboured various degrees of cognitive impairment, although with a higher proportion of moderate or severe forms (46% and 42% respectively; [table 1](#)). The cohort was highly enriched in males. Among male probands, 68% were sporadic cases, the remaining had familial history of cognitive impairment mainly evocative of an X-linked mode of

transmission ([table 1](#)). We detected certainly causative mutations in 26/106 patients ([table 2](#); see online supplementary figures S2–S19), leading to an overall diagnostic yield of 25% for the entire cohort (from 23% for sporadic cases to 31% for familial cases). Unexpectedly, the diagnostic yield appears unrelated to the severity of ID in patients ([table 1](#)).

**Table 2** List of all causative/possibly causative mutations identified in our cohort

Patient ID	Sex	Gene	Mutation	Inheritance	Mode of inheritance	Degree of ID	Consistency with classic phenotype	See online supplementary figure #
Certainly-causative mutations								
APN-58	M	<i>DYRK1A</i>	chr21:g.38858865C>T; c.613C>T; p.Arg205*; htz	De novo	AD	++	Yes	S2
APN-87	M	<i>DYRK1A</i>	chr21:g.38858873del; c.621_624delinsGAA; p.Glu208Asnfs*3; htz	De novo	AD	++	Yes	S2
APN-63	M	<i>GRIN1</i>	chr9:g.140056661C>G; c.1733C>G; p.Pro578Arg; htz	De novo	AD	+++	Yes	S3
APN-14	M	<i>MED13L</i>	chr12:g.116406845_116406852del; c.6118_6125del; p.Gly2040Asnfs*32; htz	De novo	AD	++	Partially	S4
APN-46	M	<i>RAI1</i>	chr17:g.17698594_17698598del; c.2332_2336del; p.Gly778Glnfs*7; htz	De novo	AD	++	Partially	S5
APN-122	F	<i>SHANK3</i>	chr:g.51159168_51159183dup; c.2955_2970dup; p.Pro992Argfs*325; htz	De novo	AD	+++	Yes	S6
APN-38	M	<i>SLC2A1</i>	chr1:g.43395407G>A; c.724C>T; p.Gln242*; htz	De novo	AD	+++	Yes	S7
APN-139	M	<i>SYNGAP1</i>	chr6:g.33414346G>A; c.3583-6G>A; p.Val1195Alafs*27; htz; splice	De novo	AD	++	Yes	S8
APN-41	M	<i>TCF4</i>	chr18:g.53017622_53017625del; c.514_517del; p.Lys172Phefs*61; htz	De novo	AD	+++	Yes	S9
APN-117	F	<i>TCF4</i>	chr18:g.53017619G>A; c.520C>T; p.Arg174*; htz	De novo	AD	++	No	S9
APN-138	M	<i>ATRX</i>	chrX: g.76972632G>A; c.109C>T; p.Arg37* (rs122445108); hemz	Inherited (Ma)	XL	+++	Yes	S10
APN-137	M	<i>CUL4B</i>	chrX: g.119681009_119681010del; c.811_812del; p.Gln271Aspfs*11; hemz	Inherited (Ma)	XL	+++	Partially	S11
APN-42	M	<i>DMD</i>	chrX:g.31164440del; c.10889del; p.Arg3630Glnfs*27; hemz	Inherited (Ma)	XL	++	No	S12
APN-26	M	<i>FMR1</i>	Last exon deletion; hemz	Inherited (Ma)*	XL	+++	Partially	S13
APN-113	M	<i>HCFC1</i> ; ( <i>ATRX</i> )	chrX:g.153230153G>A; c.218C>T; p.Ala73Val; hemz; (chrX:g.76939735G>C, c.1013C>G, p.Ser338Cys; hemz)	Inherited (Ma) Inherited (Ma)	XL; (XL)	+++	Yes (partially)	3
APN-82	M	<i>IL1RAPL1</i>	chrX:g.29935696_29935705del; c.894_903del; p.Trp299Thrfs*18; hemz	Inherited (Ma)	XL	++	Yes	S14
APN-68	M	<i>IQSEC2</i>	chrX:g.53268395G>A; c.3097C>T; p.Gln1033*; hemz	De novo	XL	+++	Yes	S15
APN-34	M	<i>KDM5C</i>	chrX:g.53228250C>G; c.2152G>C; p.Ala718Pro; hemz	De novo	XL	++	Partially	S16
APN-135	M	<i>KDM5C</i>	chrX:g.53240784dup; c.1296dup; p.Glu433*; hemz	Inherited (Ma)	XL	++	Partially	S16
APN-16	M	<i>MAOA</i>	chrX:g.43590942_43590943delinsTT; c.797_798delinsTT; p.Cys266Phe; hemz	Inherited (Ma)	XL	+/-	Yes	42
APN-130	F	<i>MECP2</i>	chrX: g.153296363G>A; c.916C>T; p.Arg306Cys (rs28935468); htz	De novo	XL	+++	Partially	S17
APN-142	F	<i>MECP2</i>	chrX:g.153296777G>A; c.502C>T; p.Arg168* (rs61748421); htz	De novo	XL	+++	Partially	S17
APN-3	M	<i>MECP2</i>	Complex rearrangement of exon 4; hemz	Inherited (Ma)	XL	+++	Yes	S17
APN-105	M	<i>PHF8</i> ; ( <i>DOCK8</i> )	chrX:g.54028583C>G; c.1249+5G>C; p.Tyr406Phefs*24; hemz; (chr9:g.407035G>T; c.3496G>T; p.Glu1166*; htz)	Inherited (Ma); (de novo)	XL; (AD)	+	Partially (No)	S18
APN-43	M	<i>SLC9A6</i>	chrX:g.135080258_135080262del; c.526-9_526-5del; p.?; splice disrupted; hemz	Inherited (Ma)	XL	+	Yes	Masurel-Paulet et al, in preparation
APN-110	M	<i>SLC16A2</i>	chrX:g.73749067T>C; c.1412T>C; p.Leu471Pro (rs122455132); hemz	Inherited (Ma)	XL	+++	Yes	S19

Continued

Table 2 Continued

Patient ID	Sex	Gene	Mutation	Inheritance	Mode of inheritance	Degree of ID	Consistency with classic phenotype	See online supplementary figure #
Possibly causative mutations								
APN-131	M	<i>SLC2A1</i> ; ( <i>ANKRD11</i> )	chr1:g.43392779del; c.1412delG; p.Gly471Glufs*37; htz; (chr16:g.89348867G>T; c.4083C>A; p.His1361Gln; htz)	Inherited (Pa); Inherited (Ma)	AD; AD	+++	Partially	S7
APN-101	M	<i>TCF4</i>	chr18:g.52899907C>T; c.1487-5G>A; p.Arg495_Gly496insAla?; htz	De novo ? †	AD	++	No	S9
APN-99	M	<i>NLGN3</i>	chrX:g.70389249C>T; c.1849C>T; p.Arg617Trp; hemz	Inherited (Ma)	XL	+++	Yes	S20
APN-70	M	<i>PQBP1</i>	chrX:g.48760294C>T; c.731C>T; p.Pro244Leu; hemz	Inherited (Ma)	XL	++	No	S21

\*Present in the three brothers. Mother untested, but most probably maternally inherited.

†Absent from the mother, deceased father (untested).

In bold: mutations previously reported in other patients.

–: no ID, +: mild ID, ++: moderate ID, +++: severe ID.

AD, autosomal dominant; F, female; hemz, hemizygous; htz, heterozygous; ID, intellectual disability; M, male; Ma, maternally-inherited; MAOA, Monoamine Oxidase A enzyme; Pa, paternally inherited; XL, X-linked.

Sixteen mutations are located in genes of the X-chromosome: 14 point mutations or small indels (in *ATRX*, *CUL4B*, *DMD*, *HCFC1*, *IL1RAPL1*, *IQSEC2*, *KDM5Cx2*, *MECP2x2*, *MAOA*, *PHF8*, *SLC9A6*, *SLC16A2*), as well as two larger pathogenic events (one hemizygous complex rearrangement in *MECP2*, one hemizygous exon deletion in *FMR1*; see online supplementary figures S17 and S13). We identified 10 de novo point mutations or small indels in genes involved in autosomal-dominant/haploinsufficient forms of ID (in *DYRK1Ax2*, *GRIN1*, *MED13L*, *RAI1*, *SHANK3*, *SYNGAP1*, *SLC2A1*, *TCF4x2*). In four other patients, we identified potentially causative mutations (in *NLGN3*, *PQBP1*, *SLC2A1* and *TCF4*; see online supplementary figures S20, S21, S7 and S9, respectively), whose implication in cognitive impairment has to be further confirmed. Finally, missense variants that appeared at first likely to be pathogenic, notably based on the very high evolutionary conservation of the affected residues or on previous publications, appeared excluded as causal after further segregation analysis (in *FLNA*, *FMR1*, *HUWE1* or *MECP2*, see online supplementary figure S22).

### Atypical type of mutations

Among the 26 certainly causative mutations identified, some were surprising by the nature of the mutation itself. In a boy with severe encephalopathy, epilepsy, hypotonia and microcephaly, we detected a highly complex rearrangement in exon #4 of *MECP2*, involving a 139 bp deletion flanked by the insertion of two sequences in inverted orientation derived from intron #2 still keeping the reading frame downstream of the rearrangement. Such event is inherited from the proband's mother who presents with speech delay and dyslexia (see online supplementary figure S17). This exon is known to be the one accumulating most mutations in patients, especially the 3' half, which is a recombination hotspot and the target of several deletions/duplications and less frequently inversions.<sup>16–18</sup>

We report here for the first time an intragenic deletion affecting *FMR1* outside of the promoter/exon #1 region that is the target of the fragile-X syndrome CGG-expansion. Very few point mutations have been reported in coding regions. We identified a complete deletion of the last exon of *FMR1* in one patient and his two affected brothers unevenly presenting with

clinical features of fragile-X syndrome (see online supplementary figure S13).

We also identified a patient carrying a maternally inherited 10 bp deletion causing a frameshift in exon #7 of *IL1RAPL1*, while unexpectedly his affected brother bears a de novo deletion of the full exon highlighting that affected relatives may carry distinct mutations. We propose that small 10 bp deletion may have created a sequence conformation favouring further instability and leading to the larger deletion observed in the second brother (see online supplementary figure S14). Indeed, a large proportion of *IL1RAPL1* causative mutations are intragenic exon deletions or pericentric inversions,<sup>19</sup> supporting that *IL1RAPL1* region is highly susceptible to recombination events.

Lastly, we identified a de novo Pro578Arg missense mutation in *GRIN1* in a male proband with severe ID, hypotonia, feeding disorders and very poor speech but no epilepsy, a phenotype similar to that associated to *GRIN1* missense mutations in some patients.<sup>20–21</sup> A maternal uncle presented with similar features except for the poor speech and hypotonia (see online supplementary figure S3), initially suggesting an associated X-linked mode of inheritance. The finding of a de novo deleterious missense mutation excludes this latter hypothesis, further highlighting the prevalence of phenocopies in cognitive disorders.

### Genotype–phenotype correlations: from expected to unexpected

The majority of certainly causative mutations were identified in patients whose clinical phenotype was retrospectively consistent with previous reports (table 2). For instance, the proband carrying a truncating mutation in *IQSEC2* presents with severe ID, no speech, motor developmental delay, severe epilepsy, strabismus and autistic features (see online supplementary figure S15), which matches the recently proposed clinical spectrum associated to mutations in this gene.<sup>22–23</sup> Likewise, *DYRK1A* was originally found disrupted by translocations or deleted in several patients with ID and microcephaly.<sup>24–26</sup> More recently, truncating mutations in this gene were shown to cause ID associated with primary microcephaly (sometimes borderline at –2 SD), growth retardation, developmental delay, facial dysmorphic traits, seizures and major feeding difficulties, with or without

associated autism.<sup>27–31</sup> We report here two novel de novo truncating mutations in patients with similar clinical features but no epilepsy (see online supplementary figure S2). Nonetheless, in a few other cases (eg, mutations in *RAI1* or *MECP2*), the probands lacked some clinical features, thus the corresponding diagnosis of Smith-Magenis (MIM 182290) or Rett (MIM 312750) syndrome was not evoked by experienced clinical geneticists (see online supplementary figures S5 and S17). For instance, among the three patients with *MECP2* mutations, one female had the classic Rett phenotype, the other female proband presented with non-classic Rett phenotype (no regression episode, no hand-flapping), while the rarity of reports of *MECP2* encephalopathies in adolescent males precluded suspicion of the involvement of that gene in a patient (APN-3) who had undergone rather extensive prior genetic testing.

A few detected mutations were unexpected as they were detected in patients whose phenotype did not match previous descriptions. For instance, mutations in *TCF4* mainly cause Pitt-Hopkins syndrome (PHS, MIM 610954) characterised by severe motor retardation, absence of speech, characteristic dysmorphic traits, autistic features, intestinal problems and hyperventilation.<sup>32</sup> We here describe truncating *TCF4* mutations in two patients, one with clinical manifestations highly suggestive of PHS, the other with less syndromic manifestations and no dysmorphic traits (see online supplementary figure S9). *TCF4* mutations were already reported in patients with non-syndromic ID, suggesting that such mutations were likely to be underdiagnosed.<sup>33</sup>

Another patient and his affected brother both carry a distal frameshift mutation in *DMD* affecting the major muscle transcript encoding the dystrophin protein associated to Duchenne or Becker muscular dystrophy (DMD, MIM 310200; BMD, MIM 300376), and the brain-specific isoform Dp71. The index case presents with moderate ID, psychomotor retardation, no speech, behavioural disorders, dysmorphic traits but strikingly no muscular phenotype (see online supplementary figure S12). His brother harbours a milder phenotype with additional hypotonia and cerebellar dysplasia. Both harbour borderline-high CPK levels. The association of cognitive impairment with DMD/BMD has been extensively reported and correlated to truncating mutations affecting Dp71, yet never in the absence of a muscular phenotype.<sup>34–39</sup> Our findings extend the recent report of a large family with affected males carrying an in-frame single amino acid deletion associated to mild ID and no muscular phenotype.<sup>40</sup>

### Confirmation of candidate genes for cognitive disorders

Some selected genes were only candidate ID or ASD genes at the time of the design, with single pieces of evidence in the literature. The identification of additional mutations in patients with similar phenotype definitively confirms their implication in cognitive disorders.

We reported a damaging missense affecting the function of the Monoamine Oxidase A enzyme (MAOA),<sup>41–42</sup> which replicated for the first time in 20 years the implication of MAOA in autism/ID associated to significant behavioural disorders.<sup>41–42</sup> We also identified a probably pathogenic missense variant in *NLGN3* in a male and his cousin, both presenting with ID and autism (see online supplementary figure S20). In silico predictions, high conservation of the mutated residue across all neuro-ligin paralogs, and familial analysis are altogether in favour of a pathogenicity of this missense change. A definitive functional effect of this missense still needs to be demonstrated to clearly establish the diagnosis. The implication of this gene was never

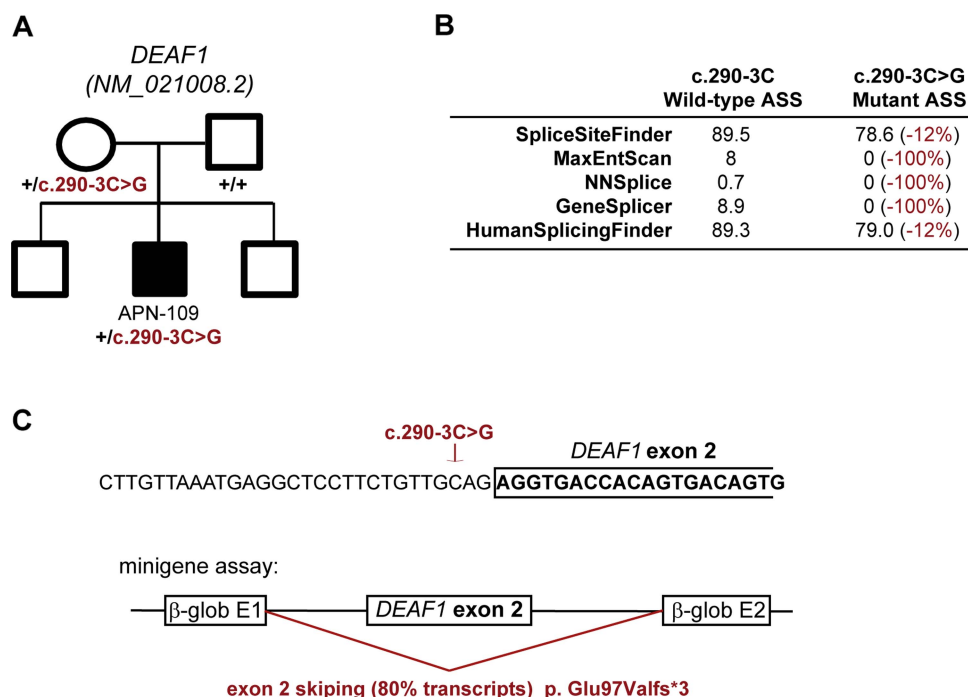
replicated since the initial publication,<sup>43</sup> although screened in several cohorts with comparable phenotypes.<sup>44–50</sup>

Similarly, we identified a novel truncating point mutation in *MED13L* confirming the implication of this gene in ID (see online supplementary figure S4). Disruption of *MED13L* was initially associated with transposition of the great arteries (TGA), associated to ID in a single case with a chromosomal translocation.<sup>51</sup> A homozygous missense mutation was then identified in two siblings from a consanguineous family presenting with non-syndromic ID, suggesting an implication of the gene in autosomal-recessive forms of ID justifying its selection in our panel.<sup>5</sup> A total of five patients were more recently described with de novo intragenic CNVs or point mutations affecting *MED13L*, delineating a recognisable *MED13L*-haploinsufficiency syndrome characterised by hypotonia, moderate ID, variable cardiac defects, facial hypotonia and dysmorphic traits.<sup>52–53</sup> Our patient with the *MED13L* mutation presents with concordant phenotype such as an open mouth appearance and muscular hypotonia but no cardiac defects. Due to the initially proposed autosomal-recessive mode of inheritance associated to ID, at first we did not consider this heterozygous truncating mutation as causative, as it may also have been the case for a heterozygous splicing mutation identified earlier in a large-scale exome sequencing study in one male with ASD.<sup>28</sup> Altogether, these findings suggest that ID associated to *MED13L*-haploinsufficiency syndrome is a relatively frequent condition.<sup>5–53</sup>

### Ambiguous mode of inheritance in ID-associated genes: the example of *DEAF1*

As for *MED13L*, the mode of inheritance associated to some genes is ambiguously described in literature. We identified an heterozygous variant affecting splicing in *DEAF1* inherited from the asymptomatic mother in a patient presenting with severe ID, developmental delay, poor speech, pain resistance, dysmorphic features and aggressive behaviour (figure 1), while the gene had been proposed as associated to autosomal-dominant forms of ID.<sup>7–8</sup> The recent report of two additional individuals carrying de novo missense mutations narrowed the associated phenotype to moderate/severe ID, speech impairment, behavioural problems, high pain threshold, dysmorphic features and abnormal walking pattern, hence highly similar to the one of our proband.<sup>54</sup> Although in vitro validation studies suggest that the reported missense variants lead to an impaired function of *DEAF1*, the authors concluded that they presumably act as dominant-negatives incapacitating both normal and mutant proteins since truncating variants had been observed in asymptomatic individuals.<sup>54</sup> In parallel, a homozygous missense mutation clustering in the same SAND-domain with all three de novo missense mutations was reported in members of a consanguineous family presenting with ID, microcephaly and white matter abnormalities, therefore suggesting a possible autosomal-recessive mode of inheritance.<sup>55</sup> The pathogenic mechanism associated to *DEAF1* mutations is therefore unclear. Due to the highly similar clinical features of the herein reported proband and of probands carrying de novo missense mutations, the splice variant detected here may contribute to the phenotype of our patient, possibly through a recessive mode of inheritance (ie, acting in *trans* with another heterozygous variant) since haploinsufficiency appears tolerated in healthy individuals. Altogether those findings either suppose a similar phenotype for autosomal-dominant and autosomal-recessive mode of inheritance associated to *DEAF1* mutations, or a universal autosomal-recessive mode of inheritance with a second variant that has not

**Figure 1** Inherited disrupting splice variant in *DEAF1*: what contribution to the phenotype? (A) Pedigree showing the maternally inherited splice variant in *DEAF1* (c.290-3C>G); (B) prediction scores for the effect of the herein described variant on splicing (prediction scores for acceptor splice sites (ASS) as computed by SpliceSite Finder, MaxEntScan, NNSplice, GeneSplicer and Human Splicing Finder for the consensus ASS with either the wild-type or the mutated allele); (C) localisation of the variant, and its resulting effect on splicing in vitro (minigene construct): 90% of abnormal transcripts: 80% with entire exon #2 skipped, and 10% using the alternative ASS c.290-16 both leading to a frameshift and a premature stop codon.



been yet identified, alike what was finally proven for Thrombocytopenia-absent radius (TAR) syndrome for instance.<sup>56</sup>

### Unsupportive evidences for proposed ID-associated mutations and genes

The identification in patients of a previously reported ID mutation should be considered with caution: indeed, as already discussed in previous publications, some 'false-positive' mutations are present among variants annotated as 'pathogenic' in dbSNP, OMIM or in published reports.<sup>13 14</sup> We here question the causative effect of a few previously proposed mutations (see online supplementary table S4). For instance, one missense variant identified in *FLNA* (c.3872C>T, p.Pro1291Leu) was previously reported in a patient with FG syndrome (MIM 300321).<sup>57</sup> This variant was identified here in a patient with a different phenotype (see online supplementary figure S22) and is also reported in one male in EVS (presumably not presenting with cognitive disorders), raising doubt about its pathogenicity.

The identification of non-segregating truncating variants (ie, detected both in patients and healthy relatives) can also challenge the implication of genes in X-linked and autosomal-dominant forms of ID. In one family, we identified a frameshift variant in *SRPX2* in a male proband. It is most likely inherited from the deceased asymptomatic maternal grandfather since it was also detected in the mother and in three maternal aunts yet absent from the maternal grandmother. Despite recent functional evidences regarding the role of *SRPX2* in brain development,<sup>58 59</sup> its definitive implication in cognitive disorders has already been questioned following the presence of the initially proposed mutations in EVS<sup>14</sup> and in control individuals,<sup>60</sup> and subsequently to the identification of a missense mutation in *GRIN2A* co-segregating with the epileptic status in the initial *SRPX2* family.<sup>61</sup> In another family, we identified a nonsense variant in *SHROOM4* in a male proband yet also in his unaffected brothers, a finding that further challenges the

implication of *SHROOM4* in X-linked cognitive disorders (see online supplementary table S5, figure 2).<sup>14</sup>

### Patients carrying probably pathogenic variants in two ID-associated genes

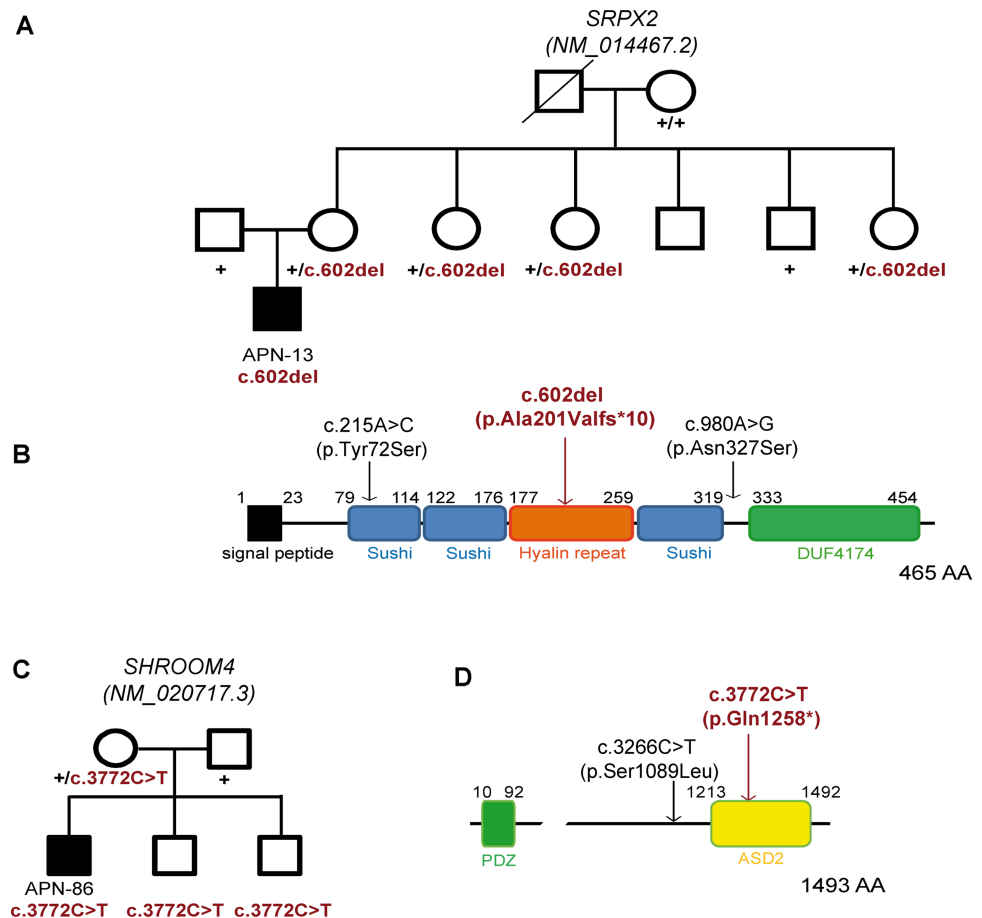
In three unrelated patients, we identified candidate variants in two separate genes, requiring the evaluation of different scenarios: (a) one single contributor while the second variant is innocuous, (b) one major contributor while the second variant acts as a modifier, and (c) both variants are implicated in the phenotype and have a synergistic effect.

In one male proband, we identified a maternally inherited splice variant leading to a frameshift in the X-linked gene *PHF8*, together with a de novo truncating variant in *DOCK8* (see online supplementary figure S18). Considering both the weak evidences implicating *DOCK8* in autosomal-dominant ID (two probands reported with translocations disrupting the gene), the clinical features consistent with a *PHF8* mutation (even in the absence of cleft lip/palate) and the X-inactivation bias identified in the mother, the *PHF8* variant alone is most likely to be responsible for the phenotype leaving the *DOCK8* variant as probably innocuous.<sup>62 63</sup>

In another male proband, two possibly causative missense variants were identified in the XLID genes *ATRX* and *HCFC1* (figure 3). Patients' phenotype was not evocative of an *ATRX* mutation (no dysmorphic traits, no urogenital abnormalities, absence of Heinz bodies), but perfectly matched the recent description of cobalamin-X metabolic disorder (*cbIX*, MIM 309541<sup>64</sup>). We concluded to a causative effect of the *HCFC1* mutation (being one of the *cbIX* recurrent mutations), but cannot exclude a contributory effect of the *ATRX* variant based on current co-segregation data.

In the third family, the proband carries one distal truncating variant in *SLC2A1* and one missense variant in *ANKRD11*, each inherited from one asymptomatic parent. Some mutations in *SLC2A1* have already been associated to GLUT-1 deficiency (MIM 606777/612126) with incomplete penetrance.<sup>65</sup> The

**Figure 2** Truncating variants not or ambiguously co-segregating with ID. (A) Pedigree of patient APN-13 carrying a frameshift variant in *SRPX2* (c.602del, p.Ala201Valfs\*10) demonstrating the likely inheritance from the asymptomatic (deceased) maternal grandfather, yet a putative germinal mosaicism of a de novo mutation cannot be excluded.; (B) predicted functional domains of *SRPX2* from Pfam indicating locations of the herein identified mutation (in red) and those previously described. DUF4174: domain of unknown function; (C) pedigree showing the non-segregating nonsense variant in *SHROOM4* (c.3772C>T; p.Gln1258\*) in the family of patient APN-86; (D) location of the premature stop codon, which would disrupt the ASD2 domain of the protein.



proband presents with evocative symptoms of both *SLC2A1* and *ANKRD11* mutations (major hypotonia along with skeletal abnormalities) that might suggest a synergistic contribution of both variants to the phenotype (see online supplementary figure S7). No conclusion could be unambiguously drawn in this case, but such a di- or oligogenic mode of inheritance has already been proposed in neurodevelopmental disorders.<sup>66–68</sup>

## DISCUSSION

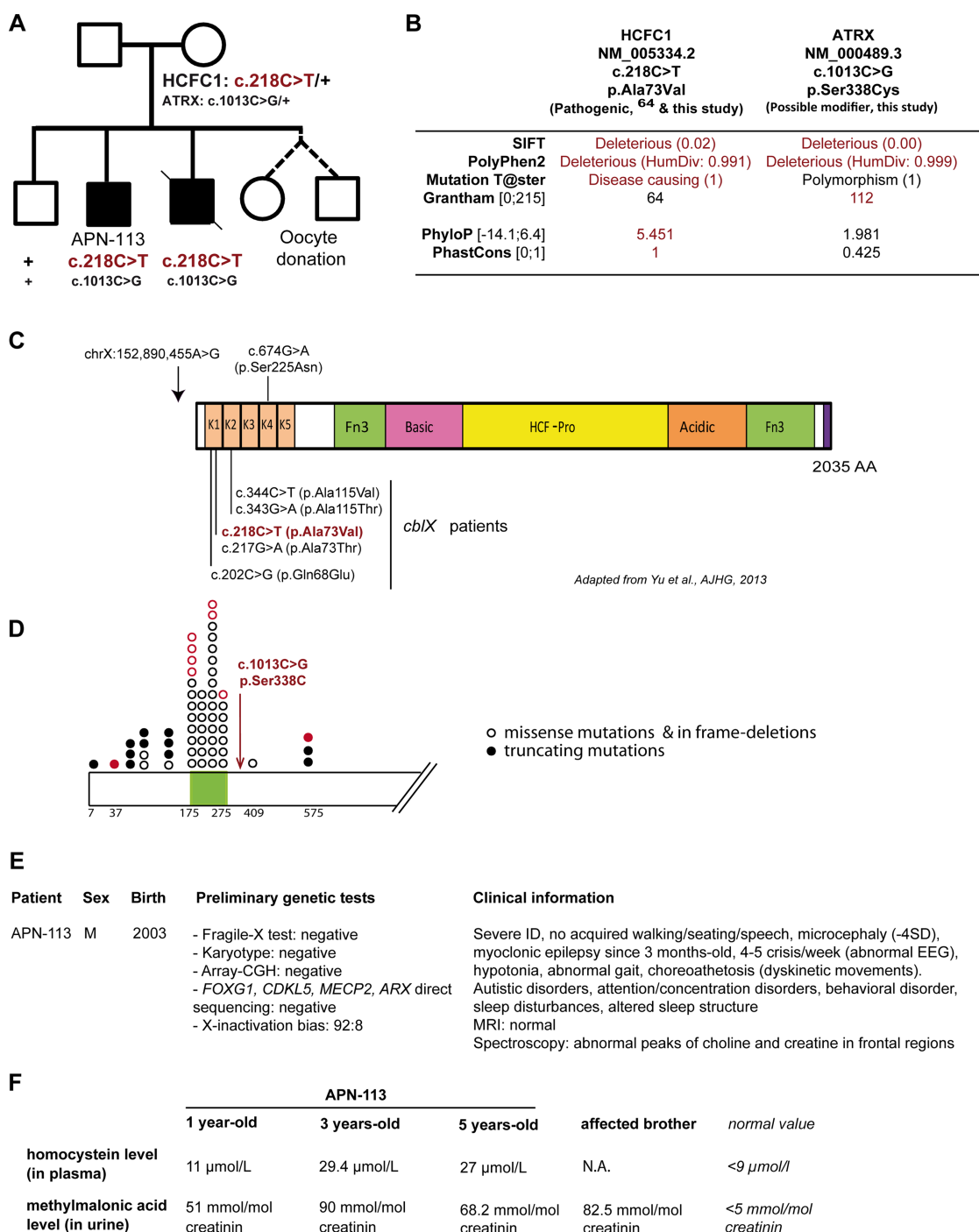
Targeted sequencing of 217 genes in a cohort of 106 patients with unknown genetic aetiology of ID led to a conclusive diagnostic yield of 25%. A majority of causative mutations identified are located in XLID genes, which can be partially explained by our male-enriched cohort. When excluding familial cases, X-linked mutations are found in 7/72 sporadic male cases, matching the proposed figure that 10% of males with sporadic ID carry mutations in X-linked genes.<sup>69</sup> Combining the results of recent trio-exome analyses leads to similar proportions (X-linked causative mutations in 7/66 males with sporadic ID<sup>3,8</sup>). The detection rate of X-linked mutations in our cohort is—as expected—higher in males with familial history of ID, but is also significant in females (2/10, both with *MECP2* mutations). Also unsurprisingly, mutations in autosomal-dominant/haploinsufficient ID genes are mostly found within sporadic cases.

Although we did not expect patients to harbour mutations in the same genes because of the small size of the cohort and the extensive genetic heterogeneity of ID, we had a few genes hit more than once: *MECP2* (despite being screened in 11% of patients prior to inclusion), *KDM5C*, *DYRK1A* and *TCF4*. Our results, intersected with the ones from other studies, highlight mutations within common genes (*MECP2*, *CUL4B*, *IL1RAPL1*,

*IQSEC2*, *KDM5C*, *SLC9A6*, *SLC16A2*, *DYRK1A*, *SLC2A1*, *SYNGAP1* or *TCF4*), suggesting that they are more frequently mutated in ID patients.<sup>3,7–9,20,28–30,70,71</sup> If mutations in such ID genes are confirmed to account for a substantial number of patients, introducing as a diagnostic step massive multiplexed resequencing of such genes in large cohorts may be considered.<sup>29</sup>

The extensive genetic and phenotypic heterogeneity of ID is the major hindrance for obtaining a precise molecular diagnosis. Direct sequencing strategies consisting in sequentially screening candidate genes are being replaced in diagnostic laboratories by more high-throughput NGS-based strategies: multiplex targeted sequencing of a few genes in large cohorts, selective targeted sequencing of up to several hundred genes, exome sequencing, while whole-genome sequencing (WGS), although powerful, notably for genome rearrangements and for CG-rich exons, remains in the research domain. Exome or full-genome strategies are more exhaustive alternatives and very attractive issues in the field of genetic diagnosis. Their universal approaches, whatever the clinical features, enhance the technical management of the workflow. However, the actual coverage proposed with the exome or full-genome strategies is still frequently insufficient (see online supplementary table S6), which may result in missing mutations.<sup>9,29</sup> Also, the finding of a putative mutation in a gene never associated before to a given pathology marks the start of a research endeavour to validate the finding but is not per se a diagnostic result.

Targeted sequencing appears more appealing for well-defined pathologies in which most implicated genes have been uncovered or in clinically homogeneous entities (ie, Bardet-Biedl and related ciliopathies,<sup>13</sup> retinal dystrophies,<sup>72</sup> hearing loss,<sup>73</sup> etc).



**Figure 3** Patient carrying two probably damaging missense variants in *HCFC1* and *ATRX*: one causative mutation and one modifier variant? (A) Family tree of patient APN-113: proband carries a missense mutation in *HCFC1* (c.218C>T; p.Ala73Val) already reported in two patients with *cbIX* as well as another missense variant in *ATRX* (c.1013C>G, p.Ser338Cys). Both variants are maternally inherited, absent from the unaffected brother, but carried by the younger brother who died of sudden death at 2 months old; (B) associated predictions for the recurrent pathogenic missense mutation in *HCFC1*<sup>64</sup> and the possible modifier in *ATRX*, showing putative pathogenicity and moderate nucleotide conservation; (C) representation of *HCFC1* and its domains: kelch domains (K1–K5), Fn3 (fibronectin type 3), basic domain, HCF-proteolysis repeats (HCF-pro), acidic domain and nuclear localisation signal (NLS) domain. The initial mutations involved in milder non-syndromic ID are indicated above: a regulatory variant in the 5'UTR of *HCFC1* was identified by targeted massive parallel resequencing in a family with probable X-linked ID (XLID) (MRX3), which had for long remained unsolved. This variant was disrupting the functional binding site of the transcription factor YY1 within the *HCFC1* promoter region, leading to an upregulation of its expression in lymphoblastoid cells.<sup>74</sup> Subsequent screening of additional unsolved families identified one single co-segregating missense variant (c.674G>A; p.Ser225Asn) in *HCFC1*. The phenotype of both patients was rather mild: non-syndromic mild to moderate ID. In the bottom are indicated the mutations recently described in *cbIX* patients<sup>64</sup>; (D) the *ATRX* missense variant is located close but outside of the hotspot for disease-causing missense mutations (in the zinc-finger binding domain, in green) reported in patients with *ATRX* mutations, in red: mutations reported independently in at least two patients. Mutations are indicated when affecting residues 1–700 (reported in OMIM, ClinVar or in<sup>75</sup>), the rest of the protein is not represented; (E) Clinical information regarding APN-113 and previous genetic explorations, including X-inactivation status in the mother. *cbIX* patients present with the same very severe phenotype: severe ID, early infantile epilepsy, choreoathetosis, microcephaly and more variable muscular hypotonia. Three of them are reported with early death in infancy<sup>64</sup>; (F) biochemical abnormalities observed in the two affected brothers are similar to those previously observed.<sup>64</sup>

We show here that it is also a powerful alternative for diagnostic purposes in ID (see online supplementary table S6). The positive diagnostic yield in our cohort of 106 patients is of 25% overall and 21% for sporadic cases, which is similar to the initial reports with the trio-exome strategy (32 highly likely causative mutations in a total of 151 patients, a 21% yield; see online supplementary table S7<sup>3,8</sup>). Indeed, most of the mutations identified by exome sequencing affect genes included in our panel and would thus have been detected with our strategy. Our diagnostic yield is slightly lower than the 30% yield (on 211 patients) we now calculated from pooling the three published exome studies (including also<sup>29</sup> and using the updated data for<sup>2</sup> very recently reported in Gilissen *et al.*<sup>9</sup>), a difference that can be accounted for by the recently recognised ID genes missing in our panel (see online supplementary table S7). Our relatively high diagnostic yield is not due to an overestimation regarding the pathogenicity of the identified variants as we were highly stringent regarding the classification of certainly causative mutations (eg, we did not include all probably damaging missense variants located in XLID genes that were detected in males). The high depth of coverage and the smaller portion of poorly covered regions achieved with our strategy ensure a high sensitivity and specificity of detecting pathogenic events in the regions of interest (SNVs, indels, but also CNVs, a topic that had not been addressed in previous exome studies). Also, the relative ease of variant analysis and smaller number of follow-up studies for candidate variants may contribute to this significant yield. As fewer candidate variants are identified per patient with this approach, they can also be analysed more thoroughly (putative effect on splicing for variants not affecting canonical splice sites, predictive impact on the protein through structural modelling, etc). With an even broader panel of genes (including, for instance, genes involved in ID associated to multiple congenital anomalies but found mutated in patients with a milder presentation), one can expect that the resulting diagnostic yield will be higher.

The limited number of sequenced genes with the targeted approach—restricted to those involved in cognitive disorders—also avoids the controversial issues raised by incidental findings. Nonetheless, the targeted sequencing approach will miss newly identified genes and theoretically prevents data reanalysis (ie, incorporating novel findings regarding genetics of ID). The major value of targeted sequencing for ID is that it should allow the application of such test to a much higher proportion of patients awaiting molecular diagnosis given the significantly lower cost of sequencing, but especially of data analysis, storage and interpretation. It should thus generate much more promptly large amounts of data regarding the spectrum of mutations and phenotypes associated to the many genes implicated in ID, and thus considerably increase our knowledge on the specific condition associated with each gene.

The substantial proportion of patients that remain without molecular diagnosis with either strategy raises several issues: whether a large fraction of ID-associated genes remain to be discovered, whether many mutations are missed because they are located in non-coding regions or lastly whether more complex genetic scenarios are implicated such as variants with reduced penetrance, oligogenic and/or multifactorial modes of inheritance.<sup>66–68</sup> In particular, many disorders associated with autosomal-dominant inheritance are associated with incomplete penetrance and high intrafamilial phenotypic variability, although such scenario is mostly excluded from trio-studies that focus on de novo mutations. The recent results from Gilissen *et al* provide initial response on this issue: a substantial

proportion of such previously negative patients seem to carry pathogenic CNVs that could not be detected by array-CGH studies (and were not tested in the exome studies), other patients carry mutations located in newly identified genes or, mutations in known genes yet that had been missed by previous bioinformatic pipelines.<sup>9</sup> With an estimated cumulative diagnostic yield of 62% (although derived from a small cohort of 50 patients, for WGS analysis), whole-genome analyses of larger cohorts of patients may shed some more light regarding the contribution of the abovementioned hypotheses to explain the remaining proportion of patients without molecular diagnosis.

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**Acknowledgements** This study was supported by grants and fellowships from Fondation pour la Recherche Médicale, Agence de Biomedecine and Fondation Jerome Lejeune, APLM and CREGEMES. We thank the students who were involved in this project: Sébastien Kirsch, Audrey Creppy, Inès Bekkour and Grace Gan. We thank Nadège Calmels, Valérie Biancalana, Elsa Nourisson and all other members of the Genetic Diagnostic Laboratory of the Nouvel Hôpital Civil (Strasbourg) for their help with patients' DNA sample selection and preparation. We thank Cecile Pizot for the development of VaRank. We thank Damien Sanlaville, Christine Coubes, Delphine Héron, Sophie Naudion, James Lespinasse and Marie-Line Bichon for their contribution to the recruitment of patients, and all medical interns or genetic counsellors who participated in this project. Finally, we warmly thank all patients and their families for their implication in this study.

**Contributors** Study concept and design: CR, J-LM, AP. Clinical genetics investigations: AM-P, MW, GL, SE-C, YA, MB, DB, EC, HD, BD, M-AD, VD-G, EF, MF, CF, AG, SL, MM-D, DM-C, DL, GM, AP, SS, CT-R, JT, MD-F, DG, PS, PE, BI and LO-F. Acquisition, analysis and interpretation of data: CR, BG, JL, YH, JM, AQ, BJ, J-LM and AP. Drafting of the manuscript: CR, J-LM and AP. Critical revision of the manuscript for important intellectual content: BG and JM. Obtained funding: J-LM and AP. Administrative, technical or material support: NH, MD, CF, VG, SLG, MP and SV. Study supervision: BG, J-LM, AP.

**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** Comité de Protection des Personnes, France.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data sharing statement** All the variants identified during the course of this study will be submitted to the variant database ClinVar: <http://www.ncbi.nlm.nih.gov/clinvar/>.

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