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Skewed X-chromosome inactivation in unsolved neurodevelopmental disease cases can guide reevaluation For X-linked genes

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1SKEWEDX-CHROMOSOMEINACTIVATIONINUNSOLVED2NEURODEVELOPMENTAL DISEASECASESCAN GUIDERE-EVALUATIONFOR X-3LINKED GENES

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- 2

54 Abstract

55 Despite major advances in genome technology and analysis, >50% of patients with a neurodevelopmental disorder (NDD) remain undiagnosed after extensive evaluation. A point in case 56 is our clinically heterogeneous cohort of NDD patients that remained undiagnosed after FRAXA 57 testing, chromosomal microarray analysis and trio exome sequencing (ES). In this study, we explored 58 the frequency of non-random X chromosome inactivation (XCI) in the mothers of male patients and 59 60 affected females, the rationale being that skewed XCI might be masking previously discarded genetic variants found on the X chromosome. A multiplex fluorescent PCR-based assay was used to analyse 61 62 the pattern of XCI after digestion with HhaI methylation-sensitive restriction enzyme. In families 63 with skewed XCI, we re-evaluated trio-based ES and identified pathogenic variants and a deletion on 64 the X chromosome. Linkage analysis and RT-PCR were used to further study the inactive X chromosome allele, and Xdrop long-DNA technology was used to define chromosome deletion 65 66 boundaries. We found skewed XCI (>90%) in 16/186 (8.6%) mothers of NDD males and in 12/90 (13.3%) NDD females, far beyond the expected rate of XCI in the normal population (3.6%, 67 OR=4.10; OR=2.51). By re-analyzing ES and clinical data, we solved 7/28 cases (25%) with skewed 68 XCI, identifying variants in KDM5C, PDZD4, PHF6, TAF1, OTUD5 and ZMYM3, and a deletion in 69 70 ATRX. We conclude that XCI profiling is a simple assay that targets a subgroup of patients that can 71 benefit from re-evaluation of X-linked variants, thus improving the diagnostic yield in NDD patients 72 and identifying new X-linked disorders.

74 INTRODUCTION

75 Major advances in exome sequencing (ES) technologies and data analysis, along with the continuing 76 identification of new disease genes, have greatly contributed to increasing the diagnostic rate of 77 neurodevelopmental disorders (NDDs). However, despite these advances, from 50-70% of NDD 78 cases remain unsolved.(1-3) Among the reasons that make molecular diagnostics challenging are: (i) 79 mutations that create unspecific phenotypes; (ii) difficulties in interpreting variants of uncertain 80 significance (VUS)(4); (iii) newly-defined diseases that describe few patients, making it difficult to 81 draw conclusions about phenotypic expansion (4); and (iv) technical limitations of the diagnostic 82 tools used.(5) Several complementary approaches can be attempted to increase the diagnostic yield 83 of unresolved NDDs, such as transcriptome analysis and whole genome sequencing. Re-analysis of 84 ES data has proven to be the most effective, increasing the diagnostic yield by 10-15%.(6)

Approximately 6% of NDDs (6% in males; 6.9% females) are estimated to be X-linked 85 86 (7)where the pathogenic genetic lesions are can often lead to non-random X-chromosome inactivation (XCI) or skewing. Although this process was identified many decades ago, the actual mechanics and 87 88 fine details of XCI have not yet been completely characterized. (8) Physiologically, XCI is random 89 and results in an approximate equal ratio of cells expressing either maternal or paternal X 90 chromosome genes. (9) XCI skewing is defined as 'preferential' (80:20%) or 'extreme' (90:10%), 91 and is a frequent indication of the presence of an X-linked pathogenetic variant, be it in affected 92 females (10) or in the mother of male patients. (11)

Females heterozygous for an X-linked pathogenetic variant are usually healthy as skewed XCI
 favors expression of the wild-type allele, thus protecting females from the deleterious effects of the
 variant.(11,12

More recently, skewed XCI has also been observed in affected females, (10) likely caused again by skewing, but this time favouring expression of a deleterious allele that reaches a pathogenicity threshold. Such female patients are susceptible to X-linked recessive conditions with a phenotype similar to that observed in male patients. (10) For X-linked dominant conditions, which 4 may be lethal in males, skewing that selects against the deleterious allele has also been observed, thus
decreasing lethality. (13)

Of the 281 undiagnosed NNDs, 276 were informative by XCI skewing (90 females + 186 mothers of males). We show that XCI skewing analysis combined with targeted re-evaluation of ES data and functional analyses can increase the diagnostic yield and identify novel X-linked disease genes.

106

We exploited the analysis of XCI skewing in 276 deeply phenotyped but undiagnosed NDD patients (90 females + 186 mothers of males). We show that XCI skewing analysis combined with targeted re-evaluation of ES data and functional analyses can increase the diagnostic yield and identify novel X-linked disease genes.

111 MATERIAL AND METHODS

112 Study cohort

From the patient cohort that is part of a large international collaborative study aimed at identifying the genetic bases of NDDs, we selected 91 affected females, and 189 mothers of affected males with negative results after trio-ES, CMA (50K Agilent) and FRAXA (see Supplemental materials and methods).

117

118 X-chromosome inactivation analysis

XCI was tested using DNA extracted from whole blood using an in-house developed protocol. The
XCI pattern was calculated using three independent microsatellite polymorphic markers on the X
chromosome : (i) the CA repeat in the promoter region of the SLIT and NTRK Like Family Member
4 (*SLITRK4*) gene; (ii) the CAG repeat located in exon 1 of the androgen receptor (*AR*) gene(14); (iii)
the CA and AG tandem repeats in the first intron of Proprotein Convertase Subtilisin/Kexin Type 1
Inhibitor (*PCSK1N*) gene (Supplemental Figure 1; supplemental materials and methods).

126 Characterization of ATRX breakpoints by indirect sequence capture coupled with Illumina 127 sequencing

128 Xdrop-based enrichment and subsequent amplification of enriched DNA was conducted at the 129 Samplix facility as previously described(15) and subsequently sequenced in 150PE on a 130 NovaSeq6000 (Illumina) (Supplemental materials and methods).

131

132 RNA extraction and RT-PCR

To determine which X-chromosome-derived allele (wt or variant) was expressed, we generated and amplified cDNA from total RNA extracted from patients' fresh blood (Supplemental materials and methods).

136

137 *X-chromosome inactivation phasing by linkage analysis*

138 Families with variants inherited from the mother and segregated in different subjects were analyzed by a set of markers to phase the identified variant with the active or inactive X chromosome and 139 140 exclude recombination events. The following genetic markers: DXS993, DXS991, DXS986, 141 DXS1068, DXS990, were amplified using AmpliTaq Gold DNA Polymerase (Thermo Fisher 142 Scientific) (see Supplemental Material and Methods for details), separated by capillary 143 electrophoresis on an ABI 3130xl DNA analyzer (Thermo Fisher Scientific) with the GeneScan 500 144 LYZ size standard (Thermo Fisher Scientific) and analyzed using the GeneMapper software v.4 145 (Thermo Fisher Scientific).

146

147 **RESULTS**

148 *X chromosome inactivation assay*

The HUMARA test, based on the analysis of a CAG repeat in the *AR* locus, is currently the gold
standard method for XCI evaluation.(14) As HUMARA is not informative in ~21% of females (due
to homozygosity or alleles of difficult interpretation), (16) we set up a fluorescent multiplex

methylation-sensitive PCR assay that simultaneously amplifies the *AR* and two additional independent polymorphic microsatellites within *SLITRK4* and *PCSK1N* (Supplemental figure 1). Firstly, we evaluated our assay in a female patient with a balanced Xq25;8q24 translocation and complete XCI skewing (100:0), previously assessed by HUMARA.(17) Complete XCI skewing was confirmed using the two additional informative loci (Supplemental table 2), demonstrating the validity of the test.

158 To further test the assay, we evaluated the XCI pattern in four females with an NDD and four healthy mothers of NDD males: all had received a previous clinical and molecular diagnosis of an X-linked 159 160 condition with potential skewed XCI (Supplemental Table 3). XCI skewing (>80%) was documented 161 in three affected females and three healthy mothers. Our findings were consistent with the literature 162 which describes the occurrence of XCI skewing in patients with pathogenetic variants in NAA10, PQBP1, MECP2, and ACSL4. (18) Similarly, we found random XCI in a healthy mother 163 164 heterozygous for a pathogenic IDS variant, which is in line with previous observations indicating occurrence of skewing in one affected female only. (18) Finally, our female patient with DDX3X had 165 a random XCI as reported for half of the patients affected by MRXSSB (MIM #300958). (18) No or 166 limited information was available in the literature for HNRNPH2, RBM10. 167

168 XCI screening in unsolved NDD cases

169 XCI screening was carried out on 281 individuals: 92 females with NDD and 189 healthy mothers of 170 males with NDD. Probands had been previously studied in depth by CMA/trio-based ES without 171 finding a genetic lesion. The phenotype of the patients varied: 35% (98/281) had intellectual disability 172 (ID), 41% (115/281) had autism spectrum disorder (ASD) and 24% (68/281) were complex 173 syndromic cases with facial dysmorphism (Supplemental Tables 4-5).

The XCI assay was informative for at least one marker in 276/281 cases (98.2%; 90/92 female
patients, 186/189 mothers) (Supplemental Tables 6-7).

- We observed 28 cases with extreme skewing (>90%) i: 12/90 female patients (13.3% vs.
 3.6%; OR=4.10; IC 95% 1.85-9.10) and 16/186 mothers (8.6% vs. 3.6%; OR=2.51; IC 95% 1.215.19) (Table1; Supplemental Figure 2-3, Supplemental Table 2).
- 179
- 180 Genetic analyses in XCI-skewed cases

To exclude the possiblility that there were genetic causes underlying the observed skewing of XCI, we sequenced the *XIST* minimal promoter in the above described 28 cases The rationale of this method was to uncover rare variants which might cause epigenetic and functional differences between X chromosomes in females as described by Plenge *et al.*(19) No such variants were found. Thus, we decided to re-evaluate the ES data, focusing on X-chromosome variants and assessing their relevance also in the light of newly available clinical data. We identified an X-linked variant consistent with the phenotype in three females and five males (8/28; 28.6%) (Table 2), as detailed below.

188

189 A novel TAF1 variant in multiple affected members of family #113

The proband was a 2-year-old girl with global developmental delay and delayed psychomotor development, and almost completely skewed XCI (95:5; Fig. 1A, Supplemental Table 2). She had a 14-year-old brother with ID, delayed speech and language development, feeding difficulties and behavioral abnormalities (II.1); a second 11-year-old brother was healthy (II.2). Their mother (I.2) reported she had had teaching support at school.

We found a c.745G>A p.(Gly249Arg) variant in Transcription initiation factor TFIID subunit 1 (*TAF1*), a gene associated with X-linked syndromic intellectual developmental disorder-33 (MRX33; MIM# 300966). MRX33 is characterized by delayed psychomotor development, ID and typical facial dysmorphisms (Supplemental Table 8).20) The variant was inherited from the mother, who also showed skewed XCI (90:10). The variant segregated with the affected brother but not with the healthy brother (Fig.1A, B). The p.(Gly249Arg) variant was absent in the GnomAD database (ver.2.1.1), and changes a highly conserved nucleotide (PhyloP= 9.37; PhastCons= 1) and amino acid 8 residue, which is maintained from vertebrates to *Drosophila melanogaster* (Fig. 1C). The change was predicted to be intolerant by MetaDome(21) (Fig 1D) and deleterious by CADD (Phred: 24.7)(22). Most of the reported likely pathogenic/pathogenic *TAF1* variants are missense substitutions that cluster between exons 16-30, whereas p.(Gly249Arg) is located in exon 6 (Fig. 1E). However, using MutScore (which takes into consideration positional clustering of variants already detected in diseaseassociated genes and variants found in the population), we noted that the variant reached a predicted pathogenicity score of 0.96 (maximum 1).(23)

The pathogenicity of p.(Gly249Arg) was also supported by the predicted structural damage triggered by disallowed phi/psi alert in Missense 3D (24) (Supplemental Material and Methods, Supplemental figure 4A).Using a series of microsatellite markers on the X chromosome (DXS993, DXS991, DXS986), we analysed the segregation of the haplotype containing the c.745G>A *TAF1* variant in the family. Because the haplotype also spanned the AR microsatellite, we could determine the c.745G>A was located on the inactive X chromosome (X_i) in both the mother (I.2) and her daughter (II.3)(Fig. 1A).

216

217 A rare de novo PHF6 variant affecting the female proband of family NWM24

218 The proband of family NWM24 was a 7-year-old girl, the second child of healthy parents. At birth, 219 she was small for gestational age (SGA), and presented global developmental delay, autistic behavior several dysmorphic features, divergent strabismus and brachy/syndactyly. XCI was completely 220 skewed (100:0)(Fig. 1F, Supplemental Table 2, Supplemental figure 6A). We identified a *de novo* 221 222 c.890G>T p.(Cys297Phe) variant in PHD finger protein 6 (PHF6; Fig. 1G), a gene associated with X-linked recessive Borjeson-Forssman-Lehmann syndrome (BFLS; MIM# 301900). We 223 224 reconsidered this previously missed variant because, in the meantime, de novo heterozygous variants have been described in affected females with an overlapping but distinct phenotype including 225 226 characteristic facial dysmorphism, dental, finger and toe abnormalities, and linear skin pigmentation 227 (Supplemental Table 9).(25, 26) These features are present in our patient. The variant is absent in 9

GnomAD (ver 2.1.1), and changes a very conserved nucleotide (PhyloP= 9.36; PhastCons= 1) and 228 229 amino acid (Fig. 1H). Cys297 is located within the PHD-like zinc-binding domain where most PHF6 pathogenic/likely pathogenic variants reported in ClinVar map (MutScore= 0.949) map. Cys297 is 230 considered intolerant to change by MetaDome (Fig. 1I; PF13771; a.a. 239-330; UniProt: Q8IWS0). 231 232 Bioinformatic analyses predict the change to be deleterious (CADD Phred= 29.5; REVEL= 0.97; Table 2). Pathogenicity of p.(Cys297Phe) was also supported by the predicted structural damage, the 233 234 amino acid substitution triggering a clash alert(24) (local clash score: wild type=10.47; mutant=35.67; 235 Supplemental Figure 4B).

236

237 A KDM5C variant with variable expressivity in family #237

In Family 237, we found a 10-year-old girl with moderate ID and skewed XCI (Fig. 1J). She was the 238 second of four siblings that included one affected brother (III.1) and two healthy sisters (III.3 and 239 240 III.4). The parents were healthy, but several male maternal relatives were reported to have ID. We found a maternally inherited c.1204G>A p.(Asp402Asn) missense variant in Lysine-specific 241 demethylase 5C (KDM5C), a gene associated with intellectual developmental disorder, X-linked, 242 syndromic, Claes-Jensen type (MRXSCJ; MIM# 300534; fig. 1K). The variant was shared by the 243 244 proband's affected brother (III.1), and one of her healthy sisters (III.3). The variant was predicted to 245 be deleterious by bioinformatic analysis (CADD Phred: 29.7; REVEL: 0.866), and the affected 246 residue mapped to a region that was considered intolerant to variation by MetaDome (Fig. 1L), and 247 conserved from vertebrates to drosophila (Fig. 1M). Another variant affecting this amino acid residue 248 [c.1204G>T p.(Asp402Tyr)] was previously demonstrated to compromise KDM5C stability and enzymatic activity.(27) 249

MRXSCJ is an X-linked recessive disorder, characterized by DD/ID with clinical heterogeneity in affected males.(28) Recurrent features include short stature, microcephaly, hyperreflexia and aggressive behavior, which were present both in the proband and her brother (III.1). Females with variants in *KDM5C*, as in case III.2, have only recently been found to be associated 10 with incomplete penetrance and a variable phenotype ranging from mild to severe ID (Supplemental
Table 10).(28) The presence of both a male and female in this family initially led us to discard Xlinked genes.

By determining the phase of AR alleles and the *KDM5C* alleles by linkage analysis, we demonstrated that the affected sister (III.2) had a preferentially active mutant allele (90%); conversely, the unaffected sister (III.3) and her mother had a preferentially inactive mutant allele (Fig. 1J).

261

262 A genomic ATRX deletion characterized by the Xdrop method in family #236

263 In family 236, the proband was a 13-year-old boy with a long diagnostic odyssey (Fig. 2A). At 3 264 years of age, he presented with hypotonia, DD/ID and dysmorphisms. The phenotype was compatible with mental retardation-hypotonic facies syndrome (MRXFH1, MIM# 309580); however, ES was 265 266 negative for an intragenic ATRX pathogenic variant (MIM* 300032). We found complete XCI skewing (100:0) in the mother, prompting us to re-evaluate the genetic data. By visually inspecting 267 the ES reads using IGV(29), we noticed no coverage of exons 3 and 4 of ATRX (Fig. 2B upper panel), 268 suggesting the presence of an intragenic deletion. According to linkage analysis, the X_i chromosome 269 270 in the mother carried the haplotype with the deletion (Fig.2A).

271 For in-depth characterization of the deletion, we used the Indirect Sequence Capture (Xdrop technology)(30,31), a powerful method for characterizing specific genomic regions. We enriched for 272 a region of ~ 100 kb within the ATRX gene, spanning the deletion. This region was subsequently 273 274 sequenced at high coverage using the Illumina NGS platform. The analysis identified the breakpoints of the deletion (Supplemental Figure 5), with an uncertainty of 3 bp, identical on both sides of the 275 276 interrupted region (hg38; chrX:77,697,545-77,703,516; chrX:77,697,542-77,703,513) (Fig. 2B). 277 Remapping of the Illumina reads on the reconstructed sequence demonstrated perfect alignment, without mismatches, thus confirming the correctness of the breakpoints (Figure 2B, lower panel). The 278

deletion of 5,971 bp was confirmed by Sanger sequencing using flanking PCR primers and shown to
be inherited from the mother (Fig. 2C, D).

281

282 PDZD4: a possible novel NDD gene in family NWM25

In family NWM25, we identified a mother of an affected boy with 90:10 XCI (Fig 2E, Supplemental Table 2). Since the age of two, the son presented symptoms of DD, followed by the development of kyphoscoliosis with pectus excavatum, hyperelastic skin and joints, persistent hand tremors, facial dysmorphisms and polymicrogyria by brain MRI. Two maternal uncles were reported to be affected by undefined ID.

Re-analysis of the X-chromosome variants led to the identification of a c.2190G>C p.(Lys736Asn) missense variant in the PDZ domain-containing 4 gene (*PDZD4*; MIM* 300634) (Fig. 2F), which was inherited from the healthy mother. Lys736 is conserved in vertebrates (Fig 2G). Using linkage analysis, we showed that the haplotype with p.(Lys736Asn) was located on the inactive Xchromosome (Fig 2E).

By exploiting GeneMatcher (https://genematcher.org/), we identified a second affected 12year-old female (II.1; family TF110, Figure 2H) with a *de novo* frameshift c.10_16del p.(Asn4Alafs*12) variant in *PDZD4*. She presented with an overlapping phenotype, including DD, microcephaly, ID and dysmorphisms. Also in this family we observed almost complete XCI (95:5) in the proband, although we could not determine if it the variant was located on the inactive X chromosome.

299

300 *OTUD5* a novel recently identified gene in family #234

The probands of family 234 were two brothers, aged 16 and 26 years, with mild ID. Their healthy
mother showed complete skewing of XCI (100:0) (Fig. 2I). We identified a missense c.1526C>T
p.(Pro509Leu) variant in OTU DOMAIN-CONTAINING PROTEIN 5 (*OTUD5*), a gene that has recently
been associated with Multiple Congenital Anomalies-Neurodevelopmental syndrome (MCAND; 12

MIM# 301056).(31) MCAND is an X-linked recessive congenital multisystemic disorder characterized by poor growth, global developmental delay with impaired intellectual development together with variable abnormalities of the cardiac, skeletal, and genitourinary systems. Disease severity is highly variable, ranging from death in early infancy to survival into the second or third decade, suggesting the variant is hypomorphic.(32)

We first confirmed that the c.1526C>T allele was indeed expressed in the patient's blood (II.1, fig. 2J). Next, we compared the cDNA sequence of *OTUD5* from the patient's blood with the *OTUD5* genomic DNA sequence (gDNA) from the mother and showed that the c.1526C>T allele was not detectable, suggesting that the skewed X inactivation preferentially silenced the chromosome with the variant (I.2, fig. 2J). Bioinformatic analyses predicted the variant to be likely pathogenic (Table 2). The substitution of leucine with proline triggers a structural damage with a local clash score of 33.58 versus a score of 15.21 calculated for the wild type protein (Supplemental Figure 4C).(24)

317

318 ZMYM3: a possible novel NDD gene in family NWM127

319 In family NWM127, subject II.1 (Fig. 2K) is a 13-year-old male with DD, moderate ID, cryptorchidism, porosis of bonesosteoporosisand dysmorphic features. He was the fourth child 320 321 in a family of European ancestry and had an affected sister (II.2) presenting with severe ID due 322 de novo tetrasomy for 15q11.2-q13.1 (MIM *608636). He was severely hypotonic in early infancy and showed relevant delay in his gross motor milestones (head control at one year and 323 sitting position at five years). He never developed fine motor skills nor acquired toilet training. 324 325 Dysmorphic features included long face, tall forehead, thick eyebrows, deeply set eyes, broad nasal tip, and low-set flashy ears with cupped formed ear lobes. Upon re-analysis of the ES data, 326 we found a c.1322G>A p.(Arg441Gln) variant in the Zinc Finger, MYM-type 3 (ZMYM3) gene. 327 The mother showed completely skewed XCI (100:0), and similar XCI skewing was found in 328 both the II.2 (90:10) and II.3 unaffected sister (85:15), with the mutant allele preferentially 329 330 inactive. Furthermore, a p.(Arg441Trp) variant was described by Philips et al. in 2014 in three

male probands with ID and several dysmorphic features shared with our proband II.1, and was
 recently confirmed as a recurrent variant in a novel ZMYM3-associated NDD.(33,34) Other
 potentially causative ES-detected variants were excluded by functional analysis [e.g., *de novo* OSBPL8: c.1535T>C; p.(Val512Ala)] that did not show altered protein activity (Prof. T. Balla,
 Bethesda, MD, *personal communication*)SION

Among the mechanisms that cause deviation from random X chromosome inactivation is selection against cells expressing X chromosomes carrying a pathogenic genetic lesion. We reasoned that we could take advantage of unbalanced XCI and use it as a guide for re-evaluating clinical and molecular data in NDD patients in which previous genetic testing failed to make a diagnosis. To test for XCI, we set up a multiplex fluorescent PCR that simultaneously analyzed the methylation status of three independent polymorphic markers on the X-chromosome. This assay allowed us to increase informativeness to >98%, compared to 80% using standard HUMARA.(14)

Analysis of 91 female NDD patients and 186 mothers of male NDD patients, previously undiagnosed by CMA and trio-ES, showed a significant enrichment of subjects with extremely unbalanced XCI, defined as a >90:10 XCI ratio (28/277, 10%) in line with the reults of a similar study.(10) The extreme skewing of XCI suggested that some of our undiagnosed cases might be attributable to a gene located on the X chromosome. Proof of principle came from the re-evaluation of available trio-ES data: by focusing on X-linked coding regions, we identified likely pathogenic variants in 7/28 cases, solving 25% of NDDs with skewed XCI.

In our original survey of 575 NDD cases, we had 28 patients with skewed XCI: nine with Xlinked variants classified as class 4 or 5, and 12 with class 3 variants. Taking into consideration these 28 cases, we estimate that X-linked genes account for 6.4-8.5% (9+28/575; 21+28/757) of the patients in our survey. Our figures are in agreement with the data from a recent evaluation of the burden of X-linked coding variation based on 11,044 Developmental Disorder patients, which estimated Xlinked causes in 6.0% of males and 6.9% of females.(7

We previously missed seven variants on the X chromosome for one of three reasons, namely: 356 357 (i) the gene was not associated with disease at the time of the analysis (OTUD5, PDZD4, and 358 ZMYM3); (ii) the variant was a structural rearrangement missed by ES (ATRX) or (iii) the variant was overlooked because it was apparently inconsistent with X-linked segregation, since both males and 359 females were affected (TAF1, PHF6, and KDM5C). In the first category, LINKage-specific-360 deubiquitylation-deficiency-induced embryonic defects (LINKED) syndrome was first associated 361 362 with pathogenic OTUD5 variants only in 2021,(32) whereas PDZD4 and ZMYM3 have been at present 363 only proposed to be disease-causing genes. Variants predicted to damage protein structure or function in ZMYM3 have been identified in patients with NDD in December 2022, (33) whereas PDZD4 has 364 365 been at present only proposed to be disease-causing gene. (35)the family with a microdeletion in 366 ATRX highlights the importance of searching for genomic rearrangements, exploiting exome data, or performing genome sequencing. In this case the deletion was missed by CMA due to lack of array 367 368 probes in the deleted tract. The strong clinical suspicion of ID-hypotonic facies syndrome (MIM# 309580) prompted us to analyze the coverage of all ATRX exons on ES data and to finally identify 369 370 the deletion of exons 3-4. We also chose to locate the precise breakpoints using a novel method based on the enrichment for targeted resequencing by the Xdrop technology, which combines high-371 372 resolution droplet PCR (dPCR) with droplet sorting and Multiple Displacement Amplification in 373 droplets (dMDA). This approach proved to be successful in fine-mapping the deletion breakpoints, 374 narrowing them down from a large putative region of ~ 20 kb between exons 2 and 5. Given the 375 flexibility of this technology, we expect it to be useful when analysing other similar cases where the 376 large size of the involved region hampers the efficient use of traditional assays for the characterization of structural variations at the single-base resolution. Alternatively, achieving the same results would 377 378 have required either genome sequencing (more expensive) or a very large sets of PCR-based assays 379 and labor/time intensive work to map the whole 20 kb region, also because the deletion maps within 380 a region rich in repeated sequences. Availability of the deletion boundaries allowed us to set up a 381 simple PCR test to follow segregation of the variant in the family.

The X chromosome is often underestimated in the diagnosis of female NDD patients because 382 383 of the common misconception that females are less susceptible to X-linked conditions.(18) Although 384 many X-linked conditions show a profound sex-linked bias, given the specific mechanism of inheritance, an increasing number of X-linked diseases have been described that occur similarly in 385 386 both female and male patients.(18) For example, in families 113 and NWM24, we identified a missense variant in TAF1 and PHF6 in a female; we overlooked/ignored these variants at the first ES 387 388 reading because inconsistent with an X-linked recessive disease. However, the literature reported females with phenotypes consistent with variants in those genes. In the case of TAF1, completely 389 390 skewed XCI is consistent with other recently described cases where the phenotype, which differs in 391 females and males, is uniform within each sex.(36) XCI unbalance favors the wild-type allele in both 392 the mother (mild phenotype) and the affected daughter, leaving the pathogenic mechanism unclear. We can speculate that: (i) expression of 5% of the pathogenic allele is sufficient to cause the 393 394 phenotype or (ii) the XCI pattern is different in affected tissues such as brain, where the pathogenic 395 allele is for some reason more expressed than in blood. In family NWM24, the phenotype associated 396 with PHF6 is consistent with the literature that reports two females carrying the de novo p.Cys305Phe, just a few amino acids distant from our proband's variant.(25, 37) 397

Family 237 is another example of X-linked gene complexity: three females carried a missense variant in *KDM5C* but we detected skewed XCI towards the deleterious allele only in the individual with the disease phenotype. Segregation analysis showed skewing towards the deleterious allele. *KDM5C* is known to escape XCI and thus the role of skewing in the phenotype is not clear.(38)

Among the various causes of female susceptibility to X-linked conditions, XCI certainly plays a key role at the penetrance level. Although the mechanism of XCI has been known for a very long time, evaluating XCI's influence on phenotype remains challenging. In some cases, the presence of skewed XCI in more easily explained by the selection of cells that inactivate the mutated allele, expressing only the wild-type allele and gaining a selective advantage during the early stages of development.(11) Typical examples are mothers heterozygous for *OTUD5*, *ATRX*, *ZMYM3* and 16 *PDZD4* variants who are protected against the deleterious effect of an X-linked pathogenetic variant by skewed XCI. In females with X-linked conditions, XCI can modulate expression of the phenotype;(39) it is likely that there are several mechanisms that underlie disease and skewing that currently escape our understanding and are not always easily identifiable. Finally, in 20 XCI-skewed cases, we could not identify any potentially causative variant. We hypothesize that the phenotype might be explained by variants in coding regions not covered by exome sequencing? or by noncoding variation, such as deep intronic variants that affect splicing or regulatory regions.

Taken together, our data conclude that XCI testing is a simple, inexpensive and productive
means for re-evaluating exome data from the X chromosome.

417

418 DATA AVAILABILITY

All variants have been deposited into ClinVar: SCV002583290, SCV002583291, SCV002583292,
SCV002583293, SCV002583294, SCV002583295, SCV002583296, SCV002583297,
SCV002583298, SCV002583299, SCV002583300, SCV002583301.

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530 AUTHOR CONTRIBUTIONS

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544 ETHICS DECLARATION

All individuals and families from the different institutions agreed to participate in this study and signed appropriate consent forms. The Ethics Committee of Città della Salute e della Scienza University Hospital (n. 0060884) and University of Skopje (n. 03-6116/7) approved this study.

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549 **DECLARATION OF INTERESTS**

550 The authors declare no competing interests.

552 **Figure legends**

Figure 1. Pedigree and variant analysis in the three families with XCI-skewed female cases. A, F, J.

Family trees of families 113, NWM24, and 237. We used X-Chromosome polymorphic microsatellites to reconstruct the haplotypes and to phase the pathogenetic variant on the inactive/active X chromosome (percentage indicated below the symbol of tested females; Xi and Xa indicate the less and the most active X chromosomes). The hyphen above each symbol indicates whenever DNA was available for genetic testing.

B, G, K. Sanger sequencing used to confirm the variants in TAF1 (NM_004606.5), PHF6 560 561 (NM_01015877.2) and KDM5C (NM_004187.5). Representative electropherograms are shown: wild type (wt); mutant hemizygous (mut); mutant heterozygous (mut/wt). C, H, M. Multiple sequence 562 563 alignment of the protein amino acid sequences in different species obtained using Marrvel software 564 for the relevant changed aminoacids (highlighted in yellow; http://marrvel.org/)(hs: Homo sapiens; mm: Mus musculus; rn: Rattus norvegicus; xt: Xenopus tropicalis; dr: Danio rerio; dm: Drosophila 565 566 melanogaster). D, I, L. Tolerance Landscape obtained using MetaDome Web Server visualizes regional tolerance to normal genetic variation (https://stuart.radboudumc.nl/metadome/). The 567 position of the missense change is indicated for each gene. The Tolerance Landscape Y-axis is 568 569 reported as a color scale from blue (position tolerant to variation, T), to yellow (position neutral to variation, N), to red (position intolerant to variation, I). Below the X- axis, a schematic representation 570 of the known protein domains (pink). E. Localization of the pathogenic (red) and likely pathogenic 571 572 (orange) variants reported in the literature for TAF1 gene in male (upper panel) and female cases 573 (lower panel). Our patient's variant is shown in black.

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575 Figure 2. Pedigrees and variants analysis in the three families with XCI skewed mothers of 576 affected males.

A, E, H, I, K. Family trees of families 236, NWM25, TF110, 234 and NWM127. See legend in figure 577 2A, F, J. B. NGS Coverage of ATRX exons (schematized above) in ES data (upper panel) and with 578 Xdrop enrichment (lower panel) in the II.1 proband from family 236. Xdrop enrichment primers (blue 579 bars below) were designed 5' of the maximum estimated deletion. After enriching DNA for the 580 region, and subsequent Illumina Sequencing, we were able to precisely identify a 5,971 bp deletion 581 spanning exons 3 and 4. C. Sanger sequencing validation of the ATRX deletion in II.1 and his mother 582 (I.2) using primers flanking the deleted segment (arrows). The deletion breakpoint is shown in panel 583 **D**. **F**, **H**. Sanger sequencing validation of the identified variants. **J**. In family 234, we sequenced the 584 genomic region (gDNA) and the corresponding transcript (cDNA) in one of the probands (II.1) and 585 their mother (I.2). The wild-type allele only was detected in both cases in the cDNA, showing that 586 the pathogenic variant was not expressed and thus located on the inactive X-chromosome. G. 587 Multiple alignment of the protein amino acid sequences in different species as described in the legend 588 589 for figure 2 C, H, M.

Table 1- Comparison of skewed X-inactivation ratio in adult population, female NDD patients and mothers of male NDD patients.

				Percentage of population with XCI							
				ratio of:							
	n.	Mean	S.D.	<80:20 >90:10 >95:5							
Adult Population *	415	52:48:00	19.3	14.2%	3.6%	1.7%					
Female NDD	90	68:32:00	14.2	22.2%	13.3%	6.7%					
Mother NDD	186	68:32:00	12.8	18.3%	8.6%	3.8%					
Notes. "Mean" indicates the mean X-chromosome inactivation (XCI) percentage within the											
group; S.D.: standard deviation. [*] Control population data obtained from ¹² .											

Family_ID	Sex	Diagnosis	MIMO	Inheritance	Gene	Variant c.DNA	Variant protein	SIFT DANN LRT Mutation Taster	ACMG Classification	XCI pattern	Evidence of X- skewing in the literature*	Escape gene**
113	F	MRXS33	300966	maternal	TAF1	NM_004606.5:c.745G>A	p.(Gly249Arg)	D 0.99 D D	Class3; PM2, PP1, PP2, PP3	100	yes	no
NWM24	F	BFLS	301900	de novo	PHF6	NM_001015877.2:c.890G>T	p.(Cys297Phe)	D 0.99 D D	Class5; PM1, PM2, PP2, PP3, PS2	90	yes	no
237	F	MRXSJ	300534	maternal	KDM5C	NM_004187.5:c.1204G>A	p.(Asp402Asn)	D 0.99 D D	Class4; PM2, PM5, PP2, PP1	90	yes	yes
234	М	MCAND	301056	maternal	OTUD5	NM_017602.4:c.1526C>T	p.(Pro509Leu)	D 0.99 N D	Class3, PM2, PP1, PP2	100	yes	no
236	М	MRXFH1	309580	maternal	ATRX	NC_000023.10:g.76953033_76959004del	p.(?)		Class5	100	yes	no
TF110	F		* 300634	de novo	PDZD4	NM_032512.5:c.10_16del	p.(Asn4Alafs*12)	/ / / /	Class4; PM2, PM4, PP3, PS2	96	N.A.	no

Table 2. Variants found in the eight families with X-skewed females

NWM25	М		* 300634	maternal	PDZD4	NM_032512.5:c.2190G>C	p.(Lys736Asn)	D 0.99 D D	Class3, PM2, PP3	91	N.A.	no
NWM127	М		* 300061	maternal	ZMYM3	NM_201599.3:c.1322G>A	p.(Arg441Gln)	T 0.99 / D	Class4, PS1, PM2	100	N.A.	no
Notes. *Data from(17); **Data from(38); D = Deleterious; N =Neutral. Variants submitted to ClinVar (SUB12130121)												







