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Expanding the phenotype of *ASXL3*-related syndrome: a comprehensive description of 45 unpublished individuals with inherited and *de novo* pathogenic variants in *ASXL3*

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CONFLICT OF INTEREST

ABSTRACT

Purpose
The study aimed at widening the clinical and genetic spectrum of *ASXL3*-related syndrome, a neurodevelopmental disorder, caused by truncating variants in the *ASXL3* gene.

Methods
In this international collaborative study, we have undertaken a detailed clinical and molecular analysis of 45 previously unpublished individuals with *ASXL3*-related syndrome, as well as a review of all previously published individuals. We have reviewed the rather limited functional characterisation of pathogenic variants in *ASXL3* and discuss current understanding of the consequences of the different *ASXL3* variants.

Results
In this comprehensive analysis of *ASXL3*-related syndrome, we define its natural history and clinical evolution occurring with age. We report familial *ASXL3* pathogenic variants, characterise the phenotype in mildly affected individuals and discuss non-penetrance. We also discuss the role of missense variants in *ASXL3*. We delineate a variable but consistent phenotype. The most characteristic features are neurodevelopmental delay with consistently limited speech, significant neuro-behavioural issues, hypotonia and feeding difficulties. Distinctive features include down-slanting palpebral fissures, hypertelorism, tubular nose with a prominent nasal bridge and low-hanging columella.

Conclusion
The presented data will inform clinical management of individuals with *ASXL3*-related syndrome and improve interpretation of new *ASXL3* sequence variants.

Key words: ASXL3, ASXL3-related syndrome, Bainbridge-Ropers syndrome, BRPS, Intellectual disability, ID, Speech impairment

INTRODUCTION

The additional sex combs-like 3 (*ASXL3* gene, MIM# 615115) was first identified as a disease-causing gene in 2013 with a report of four patients with *de novo* truncating pathogenic variants in the gene and a syndromal neurodevelopmental phenotype (Bainbridge et al., 2013). We have previously made several contributions to the literature with publication of three papers delineating the phenotype of *ASXL3*-related syndrome (Balasubramanian et al., 2017; Myers et al., 2018; Schirwani et al., 2020). So far, clinical details from a total of 45 patients with *ASXL3*-related syndrome have been reported in literature (Bainbridge et al., 2013; Balasubramanian et al., 2017; Contreras-Capetillo, Vilchis-Zapata, Ribbon-Conde, & Pinto-Escalante, 2018; Dad et al., 2017; Dinwiddie et al., 2013; Hori et al., 2016; Koboldt et al., 2018; Kuechler et al., 2017; Myers et al., 2018; Neeta Lakhani, 2017; Qiao, Liu, Ge, & Li, 2019; Schirwani et al., 2020; Srivastava et al., 2016; Verhoeven et al., 2018; Wayhelova et al., 2019). Clinically, *ASXL3*-related syndrome, also known as Bainbridge-Ropers Syndrome (BRPS, MIM# 615485), is characterised by a variable degree of intellectual disability (ID), developmental delay (DD) with absent or very limited speech development, hypotonia, feeding difficulties, behavioural problems and characteristic craniofacial features. Other findings like seizures, skeletal, palatal and dental abnormalities are common but not universal. Abnormal brain scans and Marfanoid habitus have also been described.

The ASXL family of genes consist of three members *ASXL1, ASXL2* and *ASXL3*, all of which share domains architecture, biological functions and have been implicated in human disease. The conserved domains in ASXL family consist of the ASXN, ASXH, ASXM1, ASXM2 and PHDs. The ASXH, ASXM1 and ASXM2 domains facilitate interaction of epigenetic regulators and nuclear hormone receptors. While ASXN and PHD are zinc finger domains, that play a role in regulating gene transcription (Katoh, 2015; Katoh & Katoh, 2004). The human *ASXL3* gene with its 12 exons maps to the 18q12.1 chromosome region. ASXL3 protein has 2248 amino acids (NM_030632.2; NP_085135.1), which is significantly larger than the *ASXL1* and *ASXL2* encoded proteins, highly expressed in cerebral cortex, particularly occipital cortex, ovaries, testicles and smooth muscle (Katoh, 2015; Katoh & Katoh, 2004). Studies on skin fibroblasts from individuals with truncating *ASXL3* variants have shown that ASXL3 protein is an epigenetic regulator that controls and regulates gene expression through chromatin remodeling (Katoh & Katoh, 2004; Srivastava et al., 2016). In *ASXL3* knockdown frog embryos, the early stages of neural cell fate specification including early nervous system induction and anteroposterior patterning are disturbed. These embryos also express a lower level of genes required for normal brain, primary neurons and neural crest formation (Lichtig et al., 2020).

In this report we have undertaken detailed clinical evaluation of 45 individuals with pathogenic or likely pathogenic *ASXL3* variants. We have expanded and clarified the

ASXL3-related phenotype, delineating important and recurrent clinical associations, which will inform clinical management of individuals with pathogenic variants in this gene and allow better decision-making in the context of a prenatal setting but also better health care provision for children identified early in their clinical presentation.

METHODS
Patients were identified through the Deciphering Developmental Disorders (DDD) research study(Firth, Wright, & Study, 2011) (n=14), the testing lab (GeneDx) which facilitated clinician contact (n=13), GeneMatcher (n=12), or through personal contact following publication of (Balasubramanian et al., 2017) (n=6). The numbers refer to patients we successfully obtained adequate clinical and molecular information from, which was a subset of the total number identified by each source. Individuals with variants classified as likely pathogenic or pathogenic according to ACMG classification recommendations (Richards et al., 2015), and phenotypes consistent with *ASXL3*-related syndrome were included in this study. Data was contributed by paediatric neurologists, paediatricians and, in most instances, clinical geneticists. Detailed phenotypic, molecular, cognitive and behavioural data were collected through a standardized *ASXL3* specific clinical proforma and clinical review by one of the authors. Variants were interpreted using ACMG classification recommendations (Richards et al., 2015). Genomic testing was performed in several laboratories (see supplementary information for further detail).

Ethics statement
Written informed consent for publishing clinical information was obtained from all individuals and/or families. Patients or their legal representatives have provided written consent for using images.

RESULTS
We identified 111 individuals with a clinical diagnosis of *ASXL3*-related syndrome and pathogenic or likely pathogenic variants in *ASXL3*, of which 66 were previously unpublished. Out of the 66 patients, adequate clinical information was obtained from 45, including 24 males and 21 females. The age during last assessment ranged from one month to 37 years. Patients originated from the United States, United Kingdom, Republic of Ireland, Netherlands, Spain, Italy, France, Brazil and Saudi Arabia. Cumulative prevalence of clinical features, growth parameters and milestones within this cohort is summarised in Figure 1. All numbers and percentages are related to the total number of participants for a given feature. Figure 2 A-F shows photographs of patients at various ages. Tables 1, S1 and S2 provide detailed clinical data reported in total of 90 individuals (45 previously unpublished and additional 45 reported in literature). Table S3 is an overview of features reported in one or two individuals.

Clinical data
Perinatal history
In line with previous reports, prenatal history was uneventful in most cases. The average gestational age was 39.2 weeks, with a range between 35 and 42 weeks.

Nutrition and growth

Feeding difficulty was found in 62% of individuals. Out of the seven individuals who needed long-term enteral feeding, one patient continued to require gastrostomy tube at five years of age. Feeding difficulties seem to be worst during infancy. As children got older, feeding difficulties improved, and the reflux and vomiting resolved. However, a small number of individuals continued to have recurrent vomiting, gastroesophageal reflux disease (GERD) and sensory issues related to food texture that contributed to poor nutrition. Feeding difficulties included poor suck and latch, slow feeding, recurrent vomiting, severe GERD, inability to transition to age-appropriate textured purees and solid foods, feeding refusal behaviours and a self-limiting variety of foods accepted. In eight individuals, failure to thrive (FTT) was the reason for referral.

Generally, height and weight were normal at birth, but often showed significant decline over time. At birth, only two individuals had weight below -2SD, however, measurements later in life show that 16 individuals had weight below -2SD. Seven individuals had a height of below -2SD. Occipitofrontal circumference (OFC) of below -2SD was observed in two individuals at birth. Measurements later in life showed microcephaly in 4 individuals (Figure 1). One individual's OFC dropped from -1.2SD at birth to -4 SD at 26 years of age, with MRI brain being normal. Most individuals were of slender build with normal body proportions.

Craniofacial and dental anomalies
Individuals with *ASXL3*-related syndrome share characteristic facial features comprising down-slanting palpebral fissures, ocular hypertelorism, tubular nose with a prominent nasal bridge and low hanging columella. High-arched and narrow palate, micrognathia, synophrys, deep-set eyes, long face and abnormal head shape were reported in many individuals (Figure 2A-D). Dental abnormalities were a common finding including dental over-crowding, malocclusion, large teeth to severe hypodontia, with either primary or secondary dentition missing (Figure 2E).

Musculoskeletal abnormality
The majority of individuals with *ASXL3*-related syndrome had hypotonia. Hypotonia was usually central and associated with increased tone in the upper and lower limbs. It was more prominent during the neonatal period and infancy, however, in some individuals it continued to be an issue and impacted motor development. Hypermobility was present in some individuals; one individual had a Beighton score 8/9 and another was able to put his legs behind his ears. Digital abnormalities included arachnodactyly, syndactyly, clinodactyly, contractures and tapering fingers (Figure 2E). Pectus excavatum and spinal deformity including scoliosis, kyphoscoliosis, lumbar hyperlordosis and pes planus were noted. An unusual posture and contractures were present with elbow, wrist and fingers held in flexion position (Figure 2C).

Neurodevelopment
Intellectual disability (ID)
ID of variable severity was present in all individuals who could be assessed. Severity of ID ranged from mild with one individual able to hold a part-time job, while others had profound learning difficulties and needed significant support (Figure 1).

Motor development

Gross motor skills were generally delayed in individuals with *ASXL3*-related syndrome. Motor skills ranged from normal to severely delayed, 22% sat independently at or before the age of nine months, while only 11% walked at or before 18 months of age (Figure 1). The average walking age in individuals who started walking after 18 months of age was three years old. One individual had started taking few steps using a frame at the age of six years; two individuals had not yet achieved walking at seven years and another was only able to walk with assistance at 10 years.

Speech and language development
All individuals with *ASXL3*-related syndrome had significantly delayed speech and language development. In this cohort, 48% were completely non-verbal, the age range in this group was 2-32 years with an average age of 6.6 years. In 52% of individuals, speech acquisition was achieved at an average age of 39 months; the majority had less than 10 words, while three individuals were able to speak in short sentences (Figure 1). In older non-verbal individuals, there was history of regression after acquisition of few words as toddlers. Despite absent/limited expressive language, many individuals had better receptive language skills and communicated successfully using alternative methods, such as augmentative and alternative communication (AAC).

Behavioural phenotype
A diagnosis of autism spectrum disorder (ASD) was made in 30% of individuals (Figure 1). Autistic traits including stereotypies, poor eye contact, hand flapping, rocking and head shaking, which were present in the majority of individuals. Some individuals were assessed for ASD but did not fulfil the criteria. Physical aggression was commonly reported in individuals in association with frustration and inability to express and communicate feelings. Self-injurious behaviour, including self-biting, face scratching and head banging was common too. Onset of self-injurious behaviour was as early as age two years; in other individuals, this did not start until later in life. Other behaviours included fascination with water, bruxism, inappropriate laughter, screaming, grunting and smearing faeces.

Seizures and structural brain abnormalities
Seizures were reported in 25% of individuals in this cohort. The age of onset varied from infancy to teenage years. Seizures ranged from generalized tonic-clonic seizures to absence seizures with no predominant type. Individuals with later onset epilepsy had more severe epilepsy that continued into adult life and tended to be intractable.

The electroencephalogram (EEG) findings were obtained in 12/45 individuals with reportedly normal or equivocal findings in seven individuals and abnormal findings such as multifocal generalised seizure, theta rhythmic activities, diffusely increased beta waves in five individuals. Generally, seizures responded well to standard anti-epileptic medications.

There were no characteristic findings on brain MRI, a majority of the 21% who had abnormal brain MRIs had white matter abnormalities, cerebellar vermis hypoplasia and corpus callosal abnormality (Figure 1) (Table S4 summarises the structural brain

abnormalities reported in *ASXL3*-related syndrome in the current and previously published cohorts).

Other phenotypes
A total of 10 individuals were described as having abnormal breathing patterns including apnoea, breath-holding episodes and irregular breathing patterns particularly at night, which coincided with sleep disturbances. Polysomnography results were available in three individuals, one of whom was diagnosed with moderate obstructive sleep apnoea. Recurrent ear and upper respiratory tract infections were common. Echocardiography was performed in nine individuals for various reasons and showed normal heart structure. Mild conductive hearing loss was reported in three individuals in this cohort. Cancers were not reported in our cohort.

Updated clinical information
P5 was 3.4 years and non-verbal at the time of recruitment. At six year and 10 months he has acquired 50-100 words. P11 was 10 months at the time of recruitment. At the age of four year and six months, she continues to be gastrostomy fed and unable to feed orally. She is unable to walk, non-verbal and has severe behavioural issues with self-harm and persistent crying.

Variants
In this cohort we identified 38 novel variants in 45 previously unpublished individuals. One variant was a gross deletion affecting exons 2-8, identified through chromosomal microarray (CMA). With the exception of one variant on exon three, all the other sequence variants were located in exons 11 and 12. Table 2, Table S5 and Figure 3 detail all *ASXL3* variants reported in this study as well as variants previously published in the international peer-reviewed literature (PubMed database) and Human Gene Mutation Database (HGMD professional 2019.4) (Stenson et al., 2017).

Interestingly, we observed recurrent variants in the published and our current cohort. The c.3106C>T p.(Arg1036*)(De Rubeis et al., 2014; Koboldt et al., 2018; Kosmicki et al., 2017; Kuechler et al., 2017; Myers et al., 2018) variant has been observed in six individuals, c.4330C>T p.(Arg1444*)(Balasubramanian et al., 2017; Srivastava et al., 2016) in five, c.4399C>T p.(Arg1467*) in three, and c.4534C>T p.(Gln1512*) and c.1534_1535del p.(Leu512fs) in two individuals each. Variants c.4534C>T p.(Gln1512*), c.2791_2792del p.(Gln931fs) and c.4441dup p.(Leu1481fs) were inherited, and the rest were *de novo*.

DISCUSSION
Clinical and diagnostic relevance
In the probands recruited to the DDD study, a study aimed to identify the cause of ID using large-scale exome sequencing, the prevalence of de novo variants in *ASXL3* was estimated at approximately 1/193 (50/9,625). This puts *ASXL3* among the top 10 neurodevelopmental genes for the frequency of *de novo* variants (Wright et al., 2015). Therefore, it is expected that a larger number of individuals with *ASXL3*-related syndrome will be identified with the growing use of genome/exome

sequencing for diagnosis of neurodevelopmental disorders. With rare exceptions, diagnosis of *ASXL3*-related syndrome was made through exome/genome sequencing methods rather than clinical evaluation. This could be attributed to various reasons including lack of diagnostic clues, distinctive facial features, lack of familiarity of clinicians with this specific condition and/or expanding use of genome/exome sequencing as a fist-line molecular diagnostic test.

Although the clinical diagnosis of *ASXL3*-related syndrome relies on molecular confirmation of the pathogenic variant in *ASXL3*, a distinctive phenotype is emerging and can be identified. It may not be an instantly recognisable syndrome based on facial appearance alone, but the facial features along with the history of ID, hypotonia, feeding difficulties and speech delay should prompt the clinician to consider this diagnosis. With a significant number of the more recently identified genes associated with developmental delay, one may speculate that phenotypes associated with one gene may be attributable to variants in other genes. However, facial features, albeit subtle, in addition to other features could be helpful in characterizing pathogenicity of variants and assigning causality when a variant is identified on gene panels, whole exome or whole genome sequencing. As these next generation sequencing technologies are utilized throughout the world, many additional individuals with *ASXL3*-related syndrome are likely to be identified.

This study changes the previous perception regarding *ASXL3*-related syndrome as a profound-severe neurodevelopmental disorder and highlights the prevalence of milder ID in some individuals with *ASXL3*-related syndrome. Previously, we reported an individual with apparently normal intelligence at age five years (Schirwani et al., 2020). However, when it comes to assessing cognition and behaviour, reliability is a major challenge. Most data on cognition and behaviour in the currently published individuals is based on subjective information from clinicians rather than formal testing results. Furthermore, neurobehavioral abnormalities, which are a common feature of *ASXL3*-related syndrome, may complicate the picture and impact cognitive function.

Natural history
Prenatal period and birth tend to progress normally. Feeding difficulties are present in the majority of individuals and tend to be more problematic during infancy leading to FTT in some. Development is generally delayed, particularly speech and language, with nearly half of individuals being non-verbal. Although motor delay is present in a majority of individuals, it is usually mild and tends to be made more pronounced due to a combination of hypotonia, hypermobility and FTT. The majority of individuals were referred for evaluation due to FTT, DD and hypotonia. Some individuals were not diagnosed until they were assessed later in life for behavioural problems and autism. Skeletal abnormalities, particularly digital abnormalities were common, but no major limb defects were reported. Seizure occurs in around quarter of individuals, it can be a single episode seizure, or it may continue into adulthood, and is well controlled in most individuals. Structural brain abnormalities were seen in 5/12 of brain MRIs, but other congenital anomalies (cardiac anomalies, renal anomalies and cleft palate) were very rare.

Truncating variants in *ASXL3*

The exact function of the ASXL3 protein is not well understood which resulted in some uncertainty over the mechanism through which *ASXL3* variants impacted neurodevelopment. Both dominant negative (Bainbridge et al., 2013) and a loss of function mechanism (Srivastava et al., 2016) have been proposed in this gene. Evidence from animal models suggests that truncated protein can be functional depending on the location of the truncating variants (Lichtig et al., 2020). *ASXL3* is predicted to be intolerant to protein-truncating variants with the probability of loss of function intolerance (pLI) of 1 and observed / expected (o/e) metric of 0.1. We also know that *ASXL3* knock-down frog embryos have a reduced number of primary neurons with disturbed neuronal arrangement (Lichtig et al., 2020). The last two exons of *ASXL3* (exons 11 and 12) harbour almost 98% of the variants found in this gene, consistent with their large size encompassing 83% of its coding sequence. In our study, one variant was located in exon 3 and previously a pathogenic variant was reported in exon 10 in an individual with *ASXL3*-related syndrome.(Balasubramanian et al., 2017) An *ASXL3* allele with a truncating variant in exon 12 (the last and largest exon) is expected to escape NMD, while alleles with variants within the penultimate exon (exon 11) are expected to be associated with NMD if they are located upstream of base c.2985 or codon p.(995), which is 55 bps away from the last exon/intron junction. We have decided to use the PVS1 evidence (see variant table for details) at a very strong level for all truncating variants which are expected to be associated with NMD, i.e. Located upstream of base c.2985. The PVS1 strength has been used with a one level downgrade to a strong level for truncating variants within the last exon of the gene and expecting to result in the loss of equal or larger than 10% of the protein size (Abou Tayoun et al., 2018). The cut off for a 10% loss in protein size is estimated to be at base c.6072 and codon p.(2024). Therefore, all truncating variants within the last exon and which are upstream of base c.6072 has been given a PVS1 pathogenicity evidence at a strong level (see Table 2 for more details).

Missense variants in *ASXL3*

As genomic studies become the gold standard for the identification of causative gene changes in individuals with neurodevelopmental disorders, an increasing number of missense variants are being identified. Assigning likely pathogenic/pathogenic status to missense variants continues to be challenging especially when no functional assessment of the variants is available. Genomic studies have identified missense variants in *ASXL3* in individuals with ASD in the absence of other characteristics of *ASXL3*-related syndrome (De Rubeis et al., 2014; Dinwiddie et al., 2013). Homozygous missense variants in *ASXL3* have also been implicated in *ASXL3*-related syndrome (Table S5). However, these variants classify as likely benign or variants of uncertain significance (VUS) according to ACMG guidelines, and in at least one report the patient has an alternative molecular finding, in keeping with their phenotype (Table S6).

Population data obtained from apparently typical individuals (gnomAD v2.1.1) shows constraint z score of 0.61, which indicates lower constraint and more tolerance to missense variation. The missense variants seem to be distributed throughout the gene, while the truncating variants are clustered in the last two exons of the gene. This is an area that requires further work; as more information is gathered on

missense variants, our predictive ability to interpret the clinical significance of missense changes will improve.

Penetrance
Direct estimation of penetrance of rare variants is challenging, however, the inheritance mechanism and prevalence of apparently pathogenic variants in population databases can provide some guidance. Previously published pathogenic variants in *ASXL3* were apparently *de novo* in all cases where biological parents could be tested. Although the vast majority of *ASXL3* variants identified in this study were *de novo*, three inherited variants were identified. Variants p.(Gln1512*) in P2 and p.(Gln931fs) in P10, have been inherited from affected parents and p.(Leu1481fs) in P22, has been inherited from an apparently asymptomatic mother who had a higher education qualification; there was no evidence of mosaicism in the tested blood samples. This, however, does not rule out mosaicism in these individuals as other tissues could be mosaic for the variants. Analyses of gnomAD database revealed eighteen nonsense/frameshift variants (Table S7). In three families, three non-twin siblings were found to have *de novo* pathogenic variants indicating gonadal mosaicism (Koboldt et al., 2018; Schirwani et al., 2020). We also reported an 11 year old girl with hypotonia, feeding difficulties, ID, DD, and behavioural issues who had a *de novo* pathogenic variant in *ASXL3* in 30-35% of both blood and saliva samples on trio-exome sequencing. This may indicate that only 30% mosaicism may be sufficient to cause the phenotype (Schirwani et al., 2020). Taken together, these data suggest that while the penetrance of *ASXL3* pathogenic variants appears to be high and the variants are more likely to be *de novo;* reduced- or even non-penetrance should be considered. When counselling asymptomatic parents of an affected child, *de novo* status of the variant should be confirmed prior to counselling on recurrence risk in future pregnancies.

Limitations and future prospects
Although we have undertaken a comprehensive literature review of all previously reported cases and present the largest cohort of previously unpublished individuals so far, the numbers are still small. We did not include variants that were classified as VUS classified according to the current ACMG guidelines (Richards et al., 2015), but we expect that some of these classifications may change as we learn more about the condition. Moreover, many variants are reported with very limited clinical data preventing the effort to delineate the phenotype associated with *ASXL3*-related syndrome. More information on larger groups of individuals with *ASXL3*-related syndrome is needed to determine the complete phenotypic spectrum. We have tried to account for different sources of bias when estimating various clinical features, however, it is possible that existence of some features is over or underestimated. Detailed studies of cognition, behavioral and psychiatric phenotypes through neuropsychiatric screening is crucial for all individuals with *ASXL3*-related syndrome to enable early recognition of such concerns and access to relevant support systems. Our observations suggest that children, who have not produced spoken words by the age of 6 years, are unlikely to acquire expressive language. Therefore, early introduction of intervention strategies alternative to speech is essential to improve communication.

Molecular and cellular mechanisms underlying ASXL3 protein function and the pathways through which neurodevelopment is impacted remains unclear. Studying

epi-signatures in individuals with variants in *ASXL3* may elucidate the effect of these variants on various biological pathways, cellular function and genotype-phenotype association. Identifying unique signatures associated with specific types of variants may help differentiate between benign and pathogenic variants. This will aid interpretation of the increasing number of *ASXL3* variants recognized through exome or genome sequencing, particularly in the prenatal setting (Bacrot et al., 2018).

Furthermore, all confirmed pathogenic *ASXL3* variants reported to date have been assumed or confirmed *de novo,* limiting the study of intra-familial phenotypic variability, penetrance, expressivity and recurrence risk. Until now the recurrence risk of *ASXL3*-related syndrome has been predicted to be low, albeit higher than the general population due to the possible higher risk of germline mosaicism (Koboldt et al., 2018; Schirwani et al., 2020). We emphasize the importance of parental testing prior to providing clinical recurrence risk estimates given the potential inheritance from a mildly affected or apparently asymptomatic parents.

Elucidating the role of *ASXL3* in normal development will help better understand the mechanisms through which variants in the gene may lead to neurodevelopmental disorder. It is also important to understand the variability in phenotype in individuals with *ASXL3*-related syndrome and interaction of the underlying pathogenesis in *ASXL3* with the resulting phenotype. This variability may be due to the type of variation in *ASXL3*, genetic background of the individual or environmental factors. Further research is needed to explore the function of both normal and variant ASXL3 protein. This may pave the way for exploring potential therapies that directly target *ASXL3* to replace the current symptom-guided approach for individual management.

The identification of a large number of individuals (n=45) for a rare genetic condition emphasizes the importance of data sharing, allowing delineation of clinical phenotype of *ASXL3*-related syndrome.

CONCLUSION
By presenting and analysing data from a total of 90 individuals with *ASXL3*-related syndrome, we show that *de novo* and inherited variants in *ASXL3* cause a distinctive neurodevelopmental phenotype, hallmarked by a variable degree of ID and DD with significant speech delay as a consistent feature, a variety of behavioural disorders, severe feeding difficulties, hypotonia and distinctive facial features.

REFERENCE

Abou Tayoun, A. N., Pesaran, T., DiStefano, M. T., Oza, A., Rehm, H. L., Biesecker, L. G., . . . ClinGen Sequence Variant Interpretation Working, G. (2018). Recommendations for interpreting the loss of function PVS1 ACMG/AMP variant criterion. *Hum Mutat, 39*(11), 1517-1524. doi:10.1002/humu.23626

Bacrot, S., Mechler, C., Talhi, N., Martin-Coignard, D., Roth, P., Michot, C., . . . Attie-Bitach, T. (2018). Whole exome sequencing diagnoses the first fetal case of Bainbridge-Ropers syndrome presenting as pontocerebellar hypoplasia type 1. *Birth Defects Res, 110*(6), 538-542. doi:10.1002/bdr2.1191

Bainbridge, M. N., Hu, H., Muzny, D. M., Musante, L., Lupski, J. R., Graham, B. H., . . . Ropers, H. H. (2013). De novo truncating mutations in ASXL3 are associated with a novel clinical phenotype with similarities to Bohring-Opitz syndrome. *Genome Med, 5*(2), 11. doi:10.1186/gm415

Balasubramanian, M., Willoughby, J., Fry, A. E., Weber, A., Firth, H. V., Deshpande, C., . . . Tomkins, S. (2017). Delineating the phenotypic spectrum of Bainbridge-Ropers syndrome: 12 new patients with de novo, heterozygous, loss-of-function mutations in ASXL3 and review of published literature. *J Med Genet, 54*(8), 537-543. doi:10.1136/jmedgenet-2016-104360

Contreras-Capetillo, S. N., Vilchis-Zapata, Z. H., Ribbon-Conde, J., & Pinto-Escalante, D. (2018). Global developmental delay and postnatal microcephaly: Bainbridge-Ropers syndrome with a new mutation in ASXL3. *Neurologia, 33*(7), 484-486. doi:10.1016/j.nrl.2017.01.022

Dad, R., Walker, S., Scherer, S. W., Hassan, M. J., Kang, S. Y., & Minassian, B. A. (2017). Hyperventilation-athetosis in ASXL3 deficiency (Bainbridge-Ropers) syndrome. *Neurol Genet, 3*(5), e189. doi:10.1212/NXG.0000000000000189

De Rubeis, S., He, X., Goldberg, A. P., Poultney, C. S., Samocha, K., Cicek, A. E., . . . Buxbaum, J. D. (2014). Synaptic, transcriptional and chromatin genes disrupted in autism. *Nature, 515*(7526), 209-215. doi:10.1038/nature13772

Dinwiddie, D. L., Soden, S. E., Saunders, C. J., Miller, N. A., Farrow, E. G., Smith, L. D., & Kingsmore, S. F. (2013). De novo frameshift mutation in ASXL3 in a patient with global developmental delay, microcephaly, and craniofacial anomalies. *BMC Med Genomics, 6*, 32. doi:10.1186/1755-8794-6-32

Firth, H. V., Wright, C. F., & Study, D. D. D. (2011). The Deciphering Developmental Disorders (DDD) study. *Dev Med Child Neurol, 53*(8), 702-703. doi:10.1111/j.1469-8749.2011.04032.x

Hori, I., Miya, F., Ohashi, K., Negishi, Y., Hattori, A., Ando, N., . . . Saitoh, S. (2016). Novel splicing mutation in the ASXL3 gene causing Bainbridge-Ropers syndrome. *Am J Med Genet A, 170*(7), 1863-1867. doi:10.1002/ajmg.a.37653

Katoh, M. (2015). Functional proteomics of the epigenetic regulators ASXL1, ASXL2 and ASXL3: a convergence of proteomics and epigenetics for translational medicine. *Expert Rev Proteomics, 12*(3), 317-328. doi:10.1586/14789450.2015.1033409

Katoh, M., & Katoh, M. (2004). Identification and characterization of ASXL3 gene in silico. *Int J Oncol, 24*(6), 1617-1622. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15138607

Koboldt, D. C., Mihalic Mosher, T., Kelly, B. J., Sites, E., Bartholomew, D., Hickey, S. E., . . . White, P. (2018). A de novo nonsense mutation in ASXL3 shared by siblings with Bainbridge-Ropers syndrome. *Cold Spring Harb Mol Case Stud, 4*(3). doi:10.1101/mcs.a002410

Kosmicki, J. A., Samocha, K. E., Howrigan, D. P., Sanders, S. J., Slowikowski, K., Lek, M., . . . Daly, M. J. (2017). Refining the role of de novo protein-truncating variants in neurodevelopmental disorders by using population reference samples. *Nat Genet, 49*(4), 504-510. doi:10.1038/ng.3789

Kuechler, A., Czeschik, J. C., Graf, E., Grasshoff, U., Huffmeier, U., Busa, T., . . . Wieczorek, D. (2017). Bainbridge-Ropers syndrome caused by loss-of-function variants in ASXL3: a recognizable condition. *Eur J Hum Genet, 25*(2), 183-191. doi:10.1038/ejhg.2016.165

Lichtig, H., Artamonov, A., Polevoy, H., Reid, C. D., Bielas, S. L., & Frank, D. (2020). Modeling Bainbridge-Ropers Syndrome in Xenopus laevis Embryos. *Front Physiol, 11*, 75. doi:10.3389/fphys.2020.00075

Myers, K. A., White, S. M., Mohammed, S., Metcalfe, K. A., Fry, A. E., Wraige, E., . . . Scheffer, I. E. (2018). Childhood-onset generalized epilepsy in Bainbridge-Ropers syndrome. *Epilepsy Res, 140*, 166-170. doi:10.1016/j.eplepsyres.2018.01.014

Neeta Lakhani, P. V., Chaya Kapugama and Deciphering Developmental Disorders Study DDD Study. (2017). *Milder phenotype in a boy with Bainbridge Ropers Syndrome and ASXL3 mutation. Expanding the phenotypic spectrum*. Retrieved from

Qiao, L., Liu, Y., Ge, J., & Li, T. (2019). Novel Nonsense Mutation in ASXL3 causing Bainbridge-Ropers Syndrome. *Indian Pediatr, 56*(9), 792-794.

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., . . . Committee, A. L. Q. A. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med, 17*(5), 405-424. doi:10.1038/gim.2015.30

Schirwani, S., Hauser, N., Platt, A., Punj, S., Prescott, K., Canham, N., . . . Balasubramanian, M. (2020). Mosaicism in ASXL3-related syndrome: Description of five patients from three families. *Eur J Med Genet*, 103925. doi:10.1016/j.ejmg.2020.103925

Srivastava, A., Ritesh, K. C., Tsan, Y. C., Liao, R., Su, F., Cao, X., . . . Bielas, S. L. (2016). De novo dominant ASXL3 mutations alter H2A deubiquitination and transcription in Bainbridge-Ropers syndrome. *Hum Mol Genet, 25*(3), 597-608. doi:10.1093/hmg/ddv499

Stenson, P. D., Mort, M., Ball, E. V., Evans, K., Hayden, M., Heywood, S., . . . Cooper, D. N. (2017). The Human Gene Mutation Database: towards a comprehensive repository of inherited mutation data for medical research, genetic diagnosis and next-generation sequencing studies. *Hum Genet, 136*(6), 665-677. doi:10.1007/s00439-017-1779-6

Verhoeven, W., Egger, J., Rakers, E., van Erkelens, A., Pfundt, R., & Willemsen, M. H. (2018). Phenotypic characterization of an older adult male with late-onset epilepsy and a novel mutation in ASXL3 shows overlap with the associated Bainbridge-Ropers syndrome. *Neuropsychiatr Dis Treat, 14*, 867-870. doi:10.2147/NDT.S153511

Wayhelova, M., Oppelt, J., Smetana, J., Hladilkova, E., Filkova, H., Makaturova, E., . . . Kuglik, P. (2019). Novel de novo frameshift variant in the ASXL3 gene in a child with microcephaly and global developmental delay. *Mol Med Rep, 20*(1), 505-512. doi:10.3892/mmr.2019.10303

Wright, C. F., Fitzgerald, T. W., Jones, W. D., Clayton, S., McRae, J. F., van Kogelenberg, M., . . . study, D. D. D. (2015). Genetic diagnosis of developmental disorders in the DDD study: a scalable analysis of genome-wide research data. *Lancet, 385*(9975), 1305-1314. doi:10.1016/S0140-6736(14)61705-0

FIGURE LEGENDS

Figure 1. Overview of clinical features in individuals with *ASXL3*-related syndrome.

Figure 2 A. The evolving facial appearance in six individuals with *ASXL3*-related syndrome; B. The facial appearance of adults and children with *ASXL3*-related syndrome; C. Evolving facial appearance and unusual body positioning posture with bent elbow, wrists and fingers with slight radial deviation of the hand; D. Composite facial features as generated by facial recognition technology as provided by Face2Gene in control group and individuals with *ASXL3*-related syndrome; E. Dental phenotype characterized by overcrowding, mal-alignment and severe hypodontia; F. Shows tapering finger, clinodactyly and arachnodactyly.

Figure 3 A. Distribution of *ASXL3* variants in our cohort; B. Variant types in our cohort and previously published literature. Protein domains and corresponding codons are as follows, ASXN (1-84), ASXH (250-363), ASXM1 (964-1058), ASXM2 (1741-1765).