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PUF60 **-related developmental disorder: A Case Series and phenotypic analysis of 10 additional patients with monoallelic *PUF60* variants**

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Abstract

PUF60 - related developmental disorder (also referred to as Verheij syndrome), resulting from haploinsufficiency of *PUF60* , is associated with multiple congenital anomalies affecting a wide range of body systems. These anomalies include ophthalmic coloboma, and congenital anomalies of the heart, kidney and musculoskeletal system. Behavioural and intellectual difficulties are also observed. Whilst less common than other features associated with *PUF60*-related developmental disorder, for instance hearing impairment and short stature, identification of specific anomalies such as ophthalmic coloboma can aid with diagnostic identification given the limited spectrum of genes linked with this feature. We describe 10 patients with *PUF60* gene variants, bringing the total number reported in the literature, to varying levels of details, to 56 patients.

Patients were recruited both via locally based exome sequencing from international sites and from the DDD study in the UK.

Eight of the variants reported were novel *PUF60* variants. The addition of a further patient with a reported c449-457del variant to the existing literature highlights this as a recurrent variant. One variant was inherited from an affected parent. This is the first example in the literature of an inherited variant resulting in *PUF60* -related developmental disorder.
Two patients (20%) were reported to have a renal anomaly consistent with 22% of cases in previously reported literature (see Table 2). Two patients received specialist endocrine treatment. More commonly observed were clinical features such as: cardiac anomalies (40%), ocular abnormalities (70%), intellectual disability (60%) and skeletal abnormalities (80%). Facial features did not demonstrate a recognisable gestalt. Of note, but remaining of unclear causality, we describe a single paediatric patient with pineoblastoma.

We recommend that stature and pubertal progress should be monitored in *PUF60* -related developmental disorder with a low threshold for endocrine investigations as hormone therapy may be indicated. Our study reports an inherited case with *PUF60* -related developmental disorder which has important genetic counselling implications for families.

INTRODUCTION

Pathogenic variants in the gene *PUF60* (Poly-U Binding Splicing Factor 60kDa) have been linked with key phenotypic features such as intellectual disability, coloboma and multiple congenital anomalies (5). This includes congenital anomalies of the heart, kidney and of the musculoskeletal system. With the introduction of exome sequencing for undiagnosed developmental disorders, further cases have been described. As a consequence, additional details of the *PUF60* related phenotype have been suggested, incorporating: behavioural abnormalities, gastrointestinal abnormalities, sleep disturbances and genital abnormalities(1, 28, 19).

The *PUF60* gene product is thought to influence pre-mRNA splicing and regulate transcription at the nuclear level (10). This occurs via interactions with spliceosomal proteins including SF3B4 (18). Through these interactions, snRNPs are recruited to the intron. *PUF60* is also thought to play a role in alternative splicing through promoting U2 snRNP and pre-mRNA association. (2) It is thought that *PUF60* gene function is required to ensure effective 3' splice site recognition within a specific subset of genes (2). *PUF60* function is lost in 8q24.3 deletion syndrome or *PUF60* -related developmental disorder (10). The impact of *PUF60* mRNA loss has been studied with RNA sequencing analyses and in zebrafish models. In zebrafish models of *PUF60* -related developmental disorder, features similar to the syndrome were demonstrated, with animal subjects showing reduced body length and microcephaly. (2). Until recently, it was thought that variants in both *PUF60* and its neighbouring gene, SCRIB, were required for development of *PUF60* -related developmental disorder. Recent evidence indicates that *PUF60* insufficiency alone can lead to this clinical picture. (2)

Due to the rare nature of *PUF60* -related developmental disorder, the condition's core phenotype is still progressively being characterised. Currently, 47 patients with this condition have been described to varying levels of detail in the literature. The phenotype seen in these patients is variable, however with each additional case series of this rare condition further details can be elucidated. To this body of work, we present 10 patients found to have variants in *PUF60* and their phenotypic manifestations. We expand upon one case, previously reported by Zanolli et al (30), describing this patient's phenotype in further detail.

MATERIALS AND METHODS

Patient Ascertainment

Two of the affected patients (patients 5 and patient 6 – DECIPHER IDs DDD304065 and DDD294008 respectively) were recruited from the Deciphering Developmental Disorders (DDD) study (http://www.ddduk.org) open to the UK Regional clinical genetics services (7). The remaining eight patients were recruited via locally based exome sequencing services by Clinical Geneticists in the UK, Chile and Spain. A DDD study complementary analysis ethical approval agreement was in place for the recruitment and analysis and all families gave consent for inclusion in this study.

Genomic Analysis

Trio-based exome sequencing was undertaken for the two affected patients and their parents who were identified via the DDD study(23). Four patients underwent trio based exome analysis via their local clinical genetics services in accredited laboratories using standardised techniques. The remaining three patients were tested by singleton exome sequencing analysis using a panel approach with parental testing undertaken following identification of the candidate variants.

Splicing Analysis

Analysis of the intronic variant identified in patient 7 was performed using oligonucleotide primers located within exons n-2 and n+2 from the exon of interest and a further set designed in exons further away. All primers were designed to anneal at 60°C to allow for multiple combinations within the assay. mRNA was extracted from blood from the affected parent using a PAXgene kit (Qiagen). A reverse transcription step was performed to produce cDNA, which was then amplified using a high fidelity Platinum Taq polymerase and the primers described above, which were used in combinations to achieve as much coverage of the affected region as possible and to account for possible primer failure. A normal control cDNA sample was used in each assay in order to assess assay quality. A control gene was also used to confirm the quality of the cDNA samples. PCR products were sequenced using Sanger method. Data analysis was performed using the software Mutation Surveyor. The Normal control sample was used to confirm that any variations were unique to the patient sample and not present as an alternative transcript.

Variant classification

We performed variant classification using a combination of the American College of Medical Genetics and Genomics (ACMG) guidelines (20) and a recently-developed Bayesian framework used as a quantitative tool to further refine the available evidence (23).

Patients were seen by their local clinical geneticist who obtained informed consent. Clinical details were entered into a *PUF60* phenotyping proforma.

RESULTS (See Table 1)

Demographics

Our cohort comprised five female and five male patients with an age range of 1-44years. These patients were recruited from different centres internationally: two patients were from Chile, two patients from Spain and the remainder of our cohort were recruited by clinical teams in the United Kingdom.

Clinical Features

Feeding and gastroenterology

Feeding abnormalities were present in 4 (40%) patients in our cohort. This encompassed a range of disorders of feeding, with two patients (1 and 2) requiring direct enteral feeding. Patient 1 had a poor drive for food as an infant and required a feeding tube from two until five months of age. Patient 2 was gastrostomy fed since birth and suffered from reflux. A barium meal investigation demonstrated delayed gastric emptying and dilated bowel loops. Subsequently, the patient was managed with a fundoplication.

Patients 6 and 8 experienced milder feeding difficulties that appeared to be predominantly associated with selective feeding and behavioural nuances around feeding. Patient 6 suffered from an aversion to particular food textures when in childhood and was noted to gag with food in infancy. Selective feeding was noted in patient 8 without additional gastrointestinal issues.

Patient 3 suffered from chronic diarrhoea from the age of 4 months to 4 years. Patient 9 had cow's milk protein intolerance.

Cardiac

Four patients (40%) demonstrated cardiac abnormalities. Patient 1, 2 and 5 required invasive surgical management of their cardiac defects. Patient 1 underwent surgical management for tetralogy of Fallot and experienced post-operative issues due to bronchomalacia. A cardiac anomaly was reported on Patient 2's 20-week antenatal imaging, and a diagnosis of pulmonary stenosis was confirmed on the 23 week scan. Patient 2 underwent open heart surgery for repair of pulmonary stenosis following an unsuccessful initial attempt at catheterisation of the stenosis. Tetralogy of Fallot was diagnosed in Patient 5. Patient 7 was found to have a ventriculo-septal defect in the neonatal period. It had closed within 4 months of life. He was also noted to have two small atrial septal defects. Most recent echocardiogram age 3 years and 9 months showed a small fenestrated ASD with no indication for surgical intervention. He will remain under cardiology monitoring for this.

In patient 8, whilst no severe cardiac issues were reported, a systolic murmur was noted after birth but subsequent echocardiography showed no defect.

Renal

Two patients had renal abnormalities. Patient 7 had an absent left kidney identified on renal ultrasound examination. Patient 9 had right renal agenesis, this was identified on antenatal imaging. In addition, there was a high index of suspicion that Patient 3 was likely to suffer from a renal disorder given that they developed three urinary tract infections before the age of 2 ½ years. However, the renal ultrasound was normal in this case. All 10 patients were assessed with a renal ultrasound scan to investigate for renal anomalies.

Ocular

Four patients (40 %) in our cohort had coloboma, whilst 7 had ocular abnormalities. Patient 3 had bilateral iris, retinal, optic nerve and choroidal coloboma, severe photophobia, myopia and astigmatism. Patient 4 was found to have bilateral inferior chorioretinal coloboma and strabismus. Patient 6 had right iris and chorioretinal coloboma. Left chorioretinal atrophy was found, this was optic disc sparing. Hypermetropic anisometropia with an amblyopic right eye was reported.
Patient 7 had a myopic squint, but no other ocular abnormalities. Patient 8 was also myopic. Patient 9 had low hypermetropia. Patient 10 had right sided iris and chorioretinal coloboma.

Skeletal

Although varying in severity, some form of skeletal disorder was observed in eight of the patients from this cohort.
Skeletal abnormalities involving the vertebral column were documented in patient 2, 3 and 7. The abnormalities recorded in patient 2 were a mild lower anterior vertebral beak at L1 and mild lumbar scoliosis. In patient 3, a hemivertebrae was present at L4/5.
Vertebral instability was reported at C6/7 in patient 7.
Patient 9 was found to have generalised bone age delay. The femoral heads were not yet ossified, which was later than expected given the patient's age. On investigation there was a generalised delay in epiphyseal ossification and maturation. The patient's long bones and metaphyses had a normal appearance, and bone density was also normal. Multiple metacarpal pseudoepiphyses were seen. The anterior fontanelle was large relative to the patient's age and the skull appeared large in relation to the facial bones. BoneXpert analysis was performed which demonstrated a bone age of 0.41 years at chronological age of 1.04 years, bone age z-score of -2.54. This was in-keeping with the degree of generalised epiphyseal ossification delay seen.

Patient 10 was reported as having mild chest wall asymmetry.

Abnormalities in the hands and feet were noted in patients 1, 5, 8 and 9. In patient 1, a partially reducible right clubfoot was reported. Patient 5 was found to have a hypoplastic thumb and in patient 8, the 5th toe was smaller bilaterally and mild proximal implantation of the thumbs was seen. Some skeletal abnormalities were present in Patient 6 who had flat feet, a short 5th metacarpal and joint hypermobility. Left postaxial polydactyly was observed in patient 9, arising from the left distal interphalangeal joint. Patient 10 also notably had joint hypermobility, but no other issues with their hands and feet.

Cognition and behaviour

Six patients were diagnosed with intellectual disability. Of the 4 patients without a diagnosis of intellectual disability, two patients (Patients 1 and 2) were below 2 years of age at assessment and had both experienced delays in communication and motor milestones. Patient 1 was yet to walk at 23 months and was able to speak three words at this point. Patient 2 had walked and said their first words at approximately 24 months. Patient 7 and Patient 9 were both 3.5 years old at assessment and neither were reported to experience any developmental delay.

Patient 3's level of intellectual disability was such that the patient attended mainstream secondary school education with additional educational support. The intellectual disability reported in patient 6 was such that the patient attended a Special Education Needs (SEN) unit in a mainstream secondary school. In patient 4, mild to moderate global development delay was observed at the age of 18 months. Patient 5 also required additional educational support at school given difficulties with learning disability.
Mild intellectual disability was found in patient 8, requiring an adapted school plan.
Patient 10 required a high level of additional educational support during primary education and when assessed was in the process of applying for an Education, Health and Care Plan (EHCP).

50% of our patients (5/10) experienced some form of behavioural difficulty. Behavioural difficulties were reported in Patient 2,3, 6, 7 and 10 . Patient 2 was recorded as displaying a significant level of aggressive behaviour. Behavioural difficulties were observed in Patient 6. These were self harm via hand biting, aggressive behaviour and night terrors.Temper tantrums with self injury and episodic idea fixation

were reported in patient 3. The episodic idea fixation resulted in pseudo anorexia in this case. Behavioural difficulties surrounding self harm linked with frustration were noted in Patient 7. Physically aggressive outbursts were similarly described in Patient 10.

Physical characteristics (See Figure 1)
Patient 1 had a high hairline, anteverted nostrils, thick eyebrows, high forehead, long eyelashes, thick lips and a lower eyelid crease. On antenatal assessment, nuchal translucency was seen in patient 1. Facial characteristics seen in patient 2 were upsloping palpebral fissures, epicanthic folds and a thin upper lip. Patient 3 had a high palate and was noted to have preauricular papilloma. Oral issues in patient 6 included a high palate, macrodontia and an overbite. The following physical features were seen in patient 4: turricephaly, doliocephaly, flat facial profile, short upslanting palpebral fissures, over folded helices and a short neck. Some facial features were noted in Patient 5; micrognathia, a short neck and a left ear pit. Patient 6 had a short neck, micrognathia and was found to have hyperkeratosis and multiple freckles. At birth, the patient was hirsute with different textures of hair. Patient 7 had pointed eyebrows, flat philtrum, thin upper lip and a tall square forehead. The patient was recorded as having a short neck and dry skin.
Patient 8 had a long philtrum, elongated tubular nose, wide nasal tip, long face, marked metopic suture and infraorbital folds. The patient had proportionate short stature with a relative macrocephaly.
Patient 9 was observed to have micrognathia and a long philtrum.
Patient 10 had low set, small ears with narrow canals. She also was reported to have hypertrichosis of the back and legs, a short neck, micrognathia and a long philtrum. Craniofacial asymmetry was noted in this patient.

Endocrine
6 of our 10 patients were recorded as having heights below the 10th centile for their age group (data in Table 1).
Patient 6 had small genitalia and delayed puberty and was started on testosterone therapy at the age of 15. Patient 7 had an undescended testicle. Patient 8 had proportionate short stature with a relative macrocephaly and was treated with growth hormone therapy for 3 years (Supplementary Figure 1). They have continued growth hormone therapy with demonstratable maintenance of growth velocity. There is no record of growth hormone stimulation testing being carried out prior to treatment- all endocrine results are as per the supplementary figure. Apart from with respects to growth velocity, no additional improvements in other aspects of Patient 8's condition have been reported since commencing growth hormone therapy.

Other

MRI imaging of patient 1 demonstrated volume loss and right frontotemporal microbleeds. There were no persistent neurological symptoms recorded, although one febrile seizure was reported.

In patient 2, further anomalies found on the 23 week scan included ventriculomegaly, small cerebellum and short long bones. Postnatally, the patient required phototherapy for jaundice. The patient suffered from delayed eruption of primary dentition, with no teeth emerging until after 12 months of age. Patient 2 suffered from sleep apnoea secondary to large tonsils and adenoids

Patient 3 was found to have an altered circadian rhythm which worsened with age.

Patient 4 was diagnosed with dysgenesis of the corpus callosum (rostrum and splenium) aged 20 months. At 2 years and 9 months of age she had an episode of vomiting, lethargy and seizure which resulted in her being investigated with a CT head. This showed acute hydrocephalus. Subsequent MRI was consistent with pineal tumour, haemorrhage , and obstruction of aqueduct of Silvio necessitating emergency external shunt placement. Her spine MRI showed diffuse enhancement of spinal roots, consistent with leptomeningeal dissemination and probable tethered spinal cord. Tumour biopsy showed an embryonal tumour, WHO grade IV (IHC positive for synaptophysin, chromogranin A, SMARCB1, ki 67 (in 25-30% cells). This was consistent with a diagnosis of pineoblastoma. She received chemotherapy and bone marrow transplant but recurrence of the tumour was detected about 10 months later and she died the following year.

Multiple dental caries were present in patient 5. Hypopigmented and hyperpigmented skin patches were also present.

Patient 7 developed sleep apnoea secondary to large tonsils and adenoids.
Patient 8 experienced early loss of temporary dentition and dental malposition. She had mild hearing loss. Patient 9 has macrodontia and was noted to have multiple skin tags. This patient failed the newborn hearing exam. Subsequent testing showed normal hearing.

Patient 10 required input from the incontinence team due to recurrent issues. She displayed features of autism associated with sensory differences and social communication skills at assessment. Concerns were raised about possible absence seizures and a tremor in one hand on movement in Patient 10, however all neurological investigations were normal. A double row of teeth was noted on examination. Antenatal imaging detected increased nuchal translucency in patient 10, she was also found to have ventriculomegaly at 20 weeks which resolved prior to birth.

Genetic Variants
We report 8 novel variants and two previously reported variants (Patient 3, (30) and Patient 5 (9 and 15))from 9 families. 6 truncating, 1 splice site, 1 missense and 2 in-frame deletion variants were detected. 9 were de novo in the probands and one was inherited from an affected parent, in whom the variant had arisen as a de novo event. The truncating variants were all predicted to initiate nonsense-mediated decay. With respect to the intronic variant c.818-20A>G, mRNA analysis of the variant showed aberrant splicing resulting in an out-of-frame inclusion of intron 8 (figure 2). The c.850dup p.(Val284GlyfsTer8) frameshift variant was published already in an ophthalmic

paper with minimal clinical detail and groups the patient into a group of microphthalmia, anophthalmia, coloboma (MAC) patients – the patient, previously reported by Zanolli et al (30),in fact does not have the full MAC spectrum, only coloboma (18). The c.449-457del variant seen in Patient 5 has been additionally reported in patients from the case series from Fennell et al (9) and by Kocaaga et al (15), bringing the total number of PUF-60 related developmental delay patients with this genetic variant to 3. 6 variants were classified as pathogenic according to ACMG guidelines; 3 were likely pathogenic and one is a variant of uncertain clinical significance (VUS) with a high posterior probability score. This VUS would fulfil the criteria for being classified as likely pathogenic if parentage had been confirmed, but because it was undertaken as a singleton followed by parental testing, we could only apply PM6 at supporting level.

DISCUSSION

Here, we report 9 *de novo and one inherited PUF60* variants in 10 unrelated individuals sharing common congenital anomalies associated with ID. This raises the total number of published patients with these common phenotypes harbouring *PUF60 de novo* variants to 56 and adds the first report of an inherited variant to the existing body of literature. This case series reinforces the association between variants in the *PUF60* gene and a syndrome consisting of intellectual disability and a range of congenital anomalies including; cardiac, ocular, skeletal, neurological, auditory, renal, dental and craniofacial abnormalities. Some consistency is seen between our cases and those reported (Table 2). Given the rare nature of *PUF60* -related developmental disorder, the addition of this data set – the second largest published to date- will progress efforts to delineate clinical patterns emerging from comparison of patient genotypes and phenotypes.

Two patients (20%) from our cohort (Patient 1 and 10) were found to have nuchal translucency on their antenatal scans. Toader et al (25) report an additional case of a patient with a de novo *PUF60* variant that was also noticed to have increased nuchal translucency at routine antenatal imaging. Numerous genetic syndromes have been associated previously with increased nuchal translucency, including those resulting from chromosomal abnormalities and single gene disorders (25). The link between *PUF60* and increased nuchal translucency is currently unclear; increased nuchal translucency is seen in fetuses with congenital heart disease and those with genetic disorders (4). Whilst patient 1 had tetralogy of fallot, patient 10 had no cardiac anomalies reported. This particular area requires further research.

Two of our patients were found to have significant renal anomalies. Prior to this paper, 8/36 (22.2%) of cases of *PUF60* -related developmental disorder were recorded as having renal components of their clinical presentation. Dauber et al (5) found that morpholino-mediated knockdown of either *PUF60* or Scrib in zebrafish recapitulated some of the phenotypes, including reduced body length, microcephaly, and retrognathia. Renal anomalies have also been reported in association with the 8q24.3 deletion syndrome. Previous analysis has considered that, in 8q24.3 deletion syndrome, SCRIB gene suppression (adjacent to *PUF60*) is implicated in the causation of renal defects (18).However Graziano et al (10) report agenesis of the left kidney in their patient, whilst 2 of the patients reported in the case series presented by El Chehadeh et al (8) have renal anomalies. El Chehadeh et al describe a 17 year old male patient with a phenotype including hypoplastic kidneys and a 12 year old female with a pelvic left kidney and unilateral vesicoureteral reflux (8). Similarities have been drawn previously between *PUF60* -related developmental disorder and CHARGE syndrome given the shared phenotypic features (19). Incorporating our dataset into analysis of the published cases of *PUF60* -related developmental disorder, a cumulative frequency of renal abnormalities in affected patients of 10/46 (21.7%) is seen. Given this, we would recommend that renal ultrasound should be performed as a baseline investigation following diagnosis of *PUF60*-related developmental disorder. However, given the relatively small number of patients reported it is difficult to draw strong conclusions regarding the frequency of individual features.

Patient 4 from our case series experienced seizures and hydrocephalus secondary to pineoblastoma and acute intracranial hypertension. This case is the only reported case in the literature of a patient with a *PUF60* variant developing a malignancy, therefore it is unknown if this is related to the *PUF60* variant or not. *PUF60* regulates far upstream element binding protein 1-interacting repressor (FUBP1), which is abnormally expressed in a variety of tumours and is closely involved in their progression (22). Long et al (17) describe a role of somatic *PUF60* mutations in tumorigenesis of bladder malignancy. Interestingly, it is thought that somatic overexpression of *PUF60* was associated with malignancy and more rapid tumor progression (17). In breast cancer the expression of *PUF60* was elevated in tumour tissue samples and cell lines, and its high expression was closely associated with the high incidence of lymph node metastasis and advanced TNM stage. Sun et al demonstrated that in cancer somatic *PUF60* upregulation promoted the expression of p-AKT, PI3K, and mTOR, while decreased PTEN expression through inhibiting its stability and enhancing its ubiquitination (22). *PUF60* upregulation has also been implicated in a number of other tumour types(3). In contrast, the *PUF60* -related developmental disorder patients described here and previously were found to have germline variants in *PUF60* predicted to result in haploinsufficiency, and thereforeshould result in a different downstream effect. The risk of malignancy in these patients has therefore not been considered to be increased. This single case of malignancy may be incidental but is important to record in the literature and should further cases arise this would warrant further review.

The facial features of the patients in our cohort did not fit into a consistent recognisable gestalt, consistent with previous reports (8,9, 18). The most striking facial feature is that of asymmetry which is seen in all the patients in figure 1 and was noted in previous patients (18) A number of patients do have micrognathia, but not all. Many had a thin upper lip but again this is not a consistent finding.
These findings are consistent with *PUF60* being a spliceosomal gene and part of a group of genes which cause a group of syndromes known as craniofacial spliceosomopathies (2). These genes are essential for normal neural crest cell development and as such this group of syndromes has variable degrees of abnormality of the craniofacial structures often including micrognathia, microcephaly, external ear abnormalities, malar hypoplasia and asymmetry (2). The *PUF60* knockout zebrafish described by Dauber et al (5) has craniofacial abnormalities and microcephaly. Whilst it is unlikely that these patients would be diagnosed by gestalt alone given the lack of consistency of features and lack of specificity,the astute clinician may be able to have a high index of suspicion if a patient presented with these features alongside some key phenotypes such as coloboma.

One of our patients, Patient 10, had narrow ear canals on examination. This finding is significant given previous reports of 2 patients with *PUF60* related developmental disorder also with very narrow external auditory canals (18). A further case has been reported in the literature of a patient with *PUF60* related developmental disorder with complete absence of the external auditory meatus (9). Evaluation of these previously reported cases by Low et al, suggested that *PUF60* loss of function could contribute to abnormal branchial arch development (18).

We report a patient with postaxial polydactyly, bone age delay and a generalised degree of reduced ossification. Preaxial polydactyly has been reported in a previous case series by Low et al (18). This contributes to the extended spectrum of skeletal abnormalities that can be seen with *PUF60*- related developmental disorder.

Patients with *PUF60* loss of function frequently are short in stature and report issues with growth (8). Dauber et al (5) detail the case of a patient who was managed with growth hormone therapy for slow interval growth. Their patient, a 21 year old female, had a de novo variant in *PUF60* that resulted in a single amino acid alteration that was hypothesised to have a loss of function effect on the *PUF60* protein (5). The *PUF60* knockout zebrafish described by Dauber et al demonstrated small size in comparison to wildtype. It is unclear whether this patient underwent formal growth hormone stimulation testing prior to therapy with growth hormone (5).
Patient 8 from our new series of cases, was treated with Growth Hormone therapy. Similarly, there was no evidence of formal growth hormone stimulation testing prior to therapy. In this case, growth velocity has been maintained using this therapy. Aside from this improvement with regards to growth, no other positive impact has yet been noted as a result of this treatment. Whilst these numbers are small, and clinical use of growth hormone treatment in most countries requires criteria to be met it would seem sensible for any child with a causative *PUF60* variant who has short stature to be referred to a paediatric endocrinologist for consideration of monitoring and investigation of growth if there is evidence of reduced growth velocity. Endocrinology review would also be helpful in these cases, given the description of bone age delay in one patient. Investigation of this could be considered on an individual case basis.

Other endocrine issues should also be considered in this patient group. Alkunaizhi et al (1) describe a patient presenting with Verheij syndrome who was found to have a small pituitary, with a thin stalk. This patient was not recorded as being of short stature or developing issues associated with growth and puberty. In our cohort Patient 6 was seen by paediatric endocrinology due to small genitalia and delayed puberty. He was commenced on testosterone therapy at the age of 15.
Moccia et al's report (19) describes a male patient with genital abnormalities, suggesting that this may be a recurrent issue in patients carrying causative *PUF60* variants as they progress into adulthood. Graziano et al also report a case of a male patient with genital abnormalities, in this case the patient suffered from monolateral cryptorchidism (10). Whilst short stature has been frequently commented on and explored in cases of *PUF60* deficiency, reproductive and endocrine abnormalities have not been as commonly detailed.*PUF60* is expressed throughout the genitourinary tract at both RNA and protein levels, however tissue expression is ubiquitous and non-specific throughout the body (24, 26). Genital abnormalities, particularly in male patients, may present an emerging additional phenotypic feature of this condition.

A relationship between *PUF60* -related developmental disorder and psychiatric manifestation is further suggested by our case series. In the literature previously, this has been explored to only a limited extent with behavioural difficulties only being commented on in 28 of the previously published 47 cases. Behavioural difficulties were present in 13 of the 28 cases that did consider this clinical feature. Alkhunaizi et al (1) record episodes of aggression, agitation and drop attacks in their case report. In our case series, behavioural manifestations are reported in four patients (patient 2, 3, 6 and 10). Patients 2, 6 and 10 presented with aggressive behaviour. In the case of Patient 6, this was also accompanied by behavioural issues surrounding self harm through hand biting. Patient 3, similarly to the case reported in Alkhunaizi et al (1), experienced temper tantrums that were associated with self injury. Patient 3 was recorded as experiencing an unusual psychiatric manifestation for this cohort, pseudo anorexia. In this case, the pseudoanorexia was thought to stem from an episodic idea fixation. Fennell et al (9) highlight the importance of considering psychiatric complications with this patient group and report a patient with *PUF60*- related developmental disorder who developed psychosis and was formally diagnosed with schizophrenia. Personal correspondence to Dr Karen Low has also detailed two further adults with *PUF60* -related developmental disorder with significant behaviour and psychiatric problems – unfortunately they were unable to consent to publication so no further detail can be included here. When incorporating the data from our case series, the frequency of behavioural difficulties seen in *PUF60* -related developmental disorder patients is 44.7% (17/38). The addition of our cases to the existing literature surrounding the impact of pathogenic variants in the *PUF60* gene suggests that vigilance for psychiatric manifestations in these patients, particularly in late teens and adulthood, may be prudent and early referral for support and intervention is justified. Fennell et al (9) recommend the use of standardised psychometric and neuropsychiatric assessment tools for evaluation of patient with *PUF60*. We agree, and would additionally suggest a low index of suspicion for neuropsychiatric conditions is important particularly in adolescents and young adults which should prompt early referral and intervention.

PUF60 -related developmental disorder is autosomal dominant. In the literature to date all variants have been de novo. Fennell et al (9) report two siblings that harbour the same pathogenic variant, which is suggestive of inheritance. However formal parental confirmation testing had not been completed and minimal information regarding the parents is described, and gonadal mosaicism could not be definitively excluded. This is the first report to our knowledge of an inherited variant from an affected parent (patient 7). Testing had initially been undertaken in the affected parent which identified the de novo variant - the child was subsequently tested.It has not been possible to include information regarding the affected parent. However, this documented case provides important genetic counselling information demonstrating that this is a genetic condition that is compatible with reproduction in adulthood.

The c.449_457del p.(Ala150_Phe152del) variant is located within the RNA recognition motif (RRM) of the *PUF60* protein. This variant has been previously reported in two additional cases (9,15).The other in-frame deletion (c.238_264del p.Lys80_Ile88del) and the missense

variant (c.1625T>A p.Val542Glu) do not appear to localise to any known critical domain or active site of the *PUF60* protein. However, all three variants affect evolutionarily conserved amino acid residues within regions of the protein which are significantly depleted of benign missense variation (https://www.deciphergenomics.org/gene/*PUF60* /overview/) (6). Future work on the impact of missense variants and in-frame deletions on *PUF60* function will shed more light on the pathogenic mechanism associated with these variants.

CONCLUSION

With rare syndromes such as *PUF60* -related developmental disorder, the addition and analysis of further clinical cases to the existing body of research is paramount in order to develop more precise iterations of clinical recommendations. From our analysis, we can identify several key areas for consideration when diagnosing and managing patients with *PUF60* -related developmental disorder. The frequency of renal anomalies in our cohort is broadly consistent with the frequency seen in the wider literature, given this we recommend performing renal ultrasound as a baseline investigation in all new patients.

Our case series also demonstrates a possible link between *PUF60* variants and endocrine disorders, with two of our patients (patients 6 and 8) requiring specialist treatment for hormone deficiencies (Testosterone and Growth Hormone, respectively). Consideration of endocrine disorders and, if indicated, initiating hormonal replacement therapies may have a valuable impact upon the quality of life of affected patients. As such, we recommend referral to paediatric endocrinology specialists if evidence of reduced growth velocity is observed.

Behaviour issues and psychiatric manifestations may be a considerable problem particularly in teenage years onwards and there should be a low threshold for seeking appropriate intervention and support for families with this.

The possibly incidental case of a *PUF60* variant seen in association with malignancy highlights the need a wider data set and for continued natural history studies. However, based on this single case we would not suggest routine investigation or screening in asymptomatic patients.

It is important to note the novel finding of an inherited likely pathogenic variant in *PUF60* resulting in *PUF60* -related developmental disorder in a parent and child. Testing by trio (both parents and affected child) approach is becoming a more common testing strategy but is not universal. This is therefore important information for careful genetic counselling of families and for consideration in testing strategies in the future.

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Legends:

Figure1: Photos of patients from top left to bottom right as follows: Patient 1 baby photos and aged 23 months; Patient 2; Patient 3 age 1, 18 and 20 years; Patient 5 aged 11 months and at 5 years and 4 months; Patient 6 aged 16; patient 8 aged 8; Patient 10 aged 4.5 years. Top right: sagittal views of patient 4's brain MRI T1 and T2 demonstrating a calcified, heterogeneous pineal gland mass with compression of the aqueduct of Sylvio. These were obtained at the time of diagnosis, and the tumour measured approximately 23x23x23 mm. There is moderate thinning of the corpus callosum.

Figure2: Sanger Sequencing of patient-derived cDNA from patient 7 showed aberrant splicing with inclusion of the 79bp intron 8, predicted to be out of frame. A control cDNA showing normal splicing of exons 8 and 9 is shown above the patient sequence. The genomic context of the PUF60 gene is displayed on top, with exons indicated as dark blue boxes. The location of the PUF60 intronic variant c.818-20A>G is indicated by a red arrow. Variant nomenclature and exon numbering are based on PUF60 sequence accession number NM_078480.3

Table 1: Clinical and molecular features of the 10 newly presented cases of *PUF60* -related developmental disorder.
Variant nomenclature is reported according to recommendation by HGVS (http://varnomen.hgvs.org) using GenBank accession NM_078480.3 (*PUF60* MANE Select transcript). Variant classification was performed according to ACMG guidelines and refined using posterior probabilities (in brackets) calculated by January 2018 ClinGen SVI Bayesian classification framework.

Table 2: Clinical features of *PUF60* -related developmental disorder cases previously reported in the literature compared with the new cohort of 10 cases presented.

Supplementary figure:
Clinical data collated for Patient 8 prior to and during period of Growth Hormone replacement therapy. Patient 8 is still continuing to receive this therapy, data is presented for 3 years of treatment. Growth charts from age 0-24 months (Supplementary Figure 1A) and 2-12 years (Supplementary Figure 1B), and blood test results (Supplementary Figure 1C) are reported.

AUTHOR CONTRIBUTIONS

Low K. designed the study, recruitment of study subjects, data collection and analysis, and contribution to figures and manuscript.: Grimes, H. analysis of data, literature review, first author of manuscript and tables. Ansari M, contributor to manuscript, molecular

reviewer of variants, contribution of splicing figure, review of final manuscript. Day, M, splicing studies. Calder A, skeletal radiologist who contributed data for patient. All other authors: Data collection via phenotyping proforma and review of manuscript. Supervision of project: Low K. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest. This is a statement to confirm that the authors of this paper have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Some of the data that support the findings of this study are openly available in DECIPHER: Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl at https://www.deciphergenomics.org

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