**Extending the phenotypes associated with *TRIO* gene variants in a cohort of 25 patients and review of the literature**

Gabriella Gazdagh1,2, David Hunt1, Anna Maria Cueto Gonzalez3, Monserrat Pons Rodriguez4, Ayeshah Chaudhry5,6, Marcos Madruga7, Fleur Vansenne8, Deborah Shears9, Aurore Curie10, Eva-Lena Stattin11, Britt-Marie Anderlid12, Slavica Trajkova13, Elena Sukarova Angelovska14, Catherine McWilliam15 Philip R. Wyatt16, Mary O’Driscoll17, Giles Atton1, Anke K. Bergman18, Pia Zacher19,20, Leena D Mewasingh21, Antonio Gonzalez-Meneses López22 , Olga Alonso-Luengo23 Htoo A Wai2, Ottilie Rohde2, Pauline Boiroux24, Anne Debant24, Susanne Schmidt24, Diana Baralle 2

1. Wessex Clinical Genetics Service, Princess Anne Hospital, University Hospital Southampton NHS Trust, Southampton, UK
2. Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK
3. Department of Clincial and Molecular Genetics, Vall d’Hebron Barcelona Hospital Campus, Barcelona, Spain
4. Hospital Universitari Son Espases, 07120 Palma, Illes Balears, Spain
5. Department of Laboratory Medicine and Genetics, Trillium Health Partners, Mississauga, Canada
6. Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada
7. Hospital Viamed Santa Ángela De la Cruz, Sevilla, 41014, Spain
8. Department of Clinical Genetics, University Medical Center, Groningen 9713 GZ Groningen, The Netherlands
9. Oxford Centre for Genomic Medicine, Oxford University Hospitals NHS Foundation Trust, Oxford, UK
10. Reference Center for Intellectual Disability from rare causes, Department of Child Neurology, Woman Mother and Child Hospital, Hospices Civils de Lyon, Lyon Neuroscience Research Centre, CNRS UMR5292, INSERM U1028, Université de Lyon, Bron, France
11. Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden
12. Department of molecular medisine and surgery, Karolinska Institute and Clinical Genetics, Karolinska University Hospital, Stockholm, Sweden
13. Department of Medical Sciences, Medical Genetics and Rare diseases, University of Turin, Turin, Italy
14. Department of Endocronology and Genetics, University Clinic for Children's Diseases, Medical Faculty, University Sv. Kiril i Metodij, Skopje, Republic of Macedonia
15. NHS Tayside, Ninewells Hospital, Dundee, UK
16. Department of Obstetrics and Gynecology, York Central Hospital, Toronto, Canada
17. West Midlands Regional Genetics Service, Birmingham, UK
18. Hannover Medical School, Institute of Human Genetics, Hannover, Germany
19. Epilepsy Center Kleinwachau, Radeberg, Germany
20. Institute of Human Genetics, University of Leipzig Medical Center, Leipzig, Germany
21. Department of Paediatric Neurology, Imperial College Healthcare NHS Trust, London, UK
22. Unidad de Dismorfologia, Unidad de Gestión Clínica de Pediatría, Hospital Universitario Virgen del Rocio, Sevilla, Pediatric department, University of Seville, Spain
23. Sección de Neurología Pediátrica, Unidad de Gestión Clínica de Pediatría. Hospital Universitario Virgen del Rocio, Sevilla, Pediatric department, University of Seville, Spain
24. Centre de Recherche en Biologie Cellulaire de Montpellier (CRBM), University of Montpellier, CNRS, Montpellier, France

**Address For Correspondence**

Professor Diana Baralle MD FRCP

Faculty of Medicine, University of Southampton, Human Development and Health, Southampton General Hospital

Tremona Rd, Southampton SO16 6YD

Tel 023 81204264

d.baralle@soton.ac.uk

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**Running Title**

*TRIO* gene phenotypes

**Abstract**

The *TRIO* geneencodes a Rho Guanine Exchange Factor, the function of which is to exchange GDP to GTP, and hence to activate Rho GTPases, and has been described to impact neurodevelopment. Specific genotype to phenotype correlations have been established previously describing striking differentiating features seen in variants located in specific domains of the *TRIO* gene that are associated with opposite effects on RAC1 activity. Currently, 32 cases with a *TRIO* gene alteration have been published in the medical literature. Here we report an additional 25, previously unreported individuals who possess heterozygous *TRIO* variants and we review the literature. In addition, functional studies were performed on the c.4394A>G (N1465S) and c.6244-2A>G *TRIO* variants to provide evidence for their pathogenicity. Variants reported by the current study include missense variants, truncating nonsense variants and an intragenic deletion. Clinical features were previously described and included developmental delay, learning difficulties, microcephaly, macrocephaly, seizures, behavioural issues (aggression, stereotypies), skeletal problems including short, tapering fingers and scoliosis, dental problems (overcrowding/delayed eruption) and variable facial features. Here, we report clinical features that have not been described previously, including specific structural brain malformations such as abnormalities of the corpus callosum and ventriculomegaly, additional psychological and dental issues along with a more recognisable facial gestalt linked to the specific domains of the *TRIO* gene and the effect of the variant upon the function of the encoded protein. This current study further strengthens the genotype to phenotype correlation that was previously established and extends the range of phenotypes, to include structural brain abnormalities, additional skeletal, dental andpsychiatric issues.

**Introduction**

Guanine exchange factors (GEFs), such as TRIO, are known to activate Rho guanosine triphosphatases (Rho GTPases) by facilitating the guanosine diphosphate (GDP)-guanosine triphosphate (GTP) exchange. Rho GTPases play a central part in neurodevelopment as they have a central regulatory role in actin cytoskeleton dynamics (Govek et al., 2005; Paskus et al., 2020; Tolias et al., 2011). TRIO is highly expressed in the developing brain (Ba et al., 2016; McPherson et al., 2005; Paskus et al., 2020; Portales-Casamar et al., 2006) and it is well-recognised to have a major impact on several important processes including cell migration, axonal outgrowth and dendritic arborization and synaptogenesis (Ba et al., 2013; Briancon-Marjollet et al., 2008; Herring & Nicoll, 2016; Iyer et al., 2012; Schmidt & Debant, 2014). TRIO is highly conserved across the species and comprises of two GEF domains and accessory motifs such as the spectrin repeats (Debant et al., 1996). The first GEF domain, GEFD1, modulates RAC1 and RHOG while the second GEF domain, GEFD2 regulates RHOA (Bellanger et al., 1998; Blangy et al., 2000).

Previous studies have described *TRIO* variants in individuals with neurodevelopmental disorders and associated features included macrocephaly, microcephaly, behaviour problems, seizures, gastrointestinal problems, skeletal features, dental problems, hyperactivity, aggression, autism spectrum disorder, and hand anomalies amongst others (Ba et al., 2016; Barbosa et al., 2020; Pengelly et al., 2016; Sadybekov et al., 2017).

*TRIO* gene missense variants have been shown to cluster in two main mutational hotspots, namely the spectrin and GEFD1 domains, which are linked to two distinct phenotypes. Pathogenic and likely pathogenic variants within the spectrin domain have been associated with more severe developmental issues and macrocephaly, via TRIO-induced RAC1 hyperactivity and a gain-of-function mechanism, whereas variants in the GEFD1 domain were shown to lead to a milder phenotype with less pronounced neurodevelopmenal delay and microcephaly, with demonstratable reduced activity of the TRIO protein and a loss-of-function mechanism (Barbosa et al., 2020).

Here we describe detailed phenotype information of an additional 25 patients and review previously published cases. The current study extends the spectrum of phenotypes with additional skeletal features, dental anomalies and structural brain malformations. In addition, psychiatric issues including bipolar disorders and anxiety are described.

This is the largest cohort of patients with a *TRIO* variant to date, with a potential to extend and refine the phenotypic associations with mutations in this gene.

**Patients & Methods**

Patients and families wishing to participate in the current project contacted our team via a patient support group called Team TRIO, ([www.teamtrio.org)](http://www.teamtrio.org)b) between July 2021 and August 2022. We also included cases referred to us by clinicians directly or via existing collaboration with other research teams and patient databases including the DECIPHER, Matchmaker and the Human Disease Genes Database. Clinicians and participating families were asked to complete a clinical data collection form and provide photographs of the affected individual. Consent for publication was obtained from all patients and their legal guardians conforming to the standards of the Declaration of Helsinki.

Phenotype and genotype data of individuals with a reported pathogenic or likely pathogenic *TRIO* gene variant have been analysed and photographs (face, hands and feet) have been collated to delineate facial and limb features. The only exception were patients 14 and 15 whose variants [c.4394A>G, p.(Asn1465Ser)] were classed as variants of uncertain significance (VUS), and patient 23 with the splice site variant c.6244-2A>G, therefore we endeavoured to perform functional studies detailed below to provide evidence for their pathogenicity.

**Functional study on TRIO variant N1465S**

HEK293T and N1E-115 neuroblastoma cells were cultured and transfected as described in Barbosa et al, 2020. Immunoblot analysis for quantification of Phospho-PAK levels were also performed as described in Barbosa et al, 2020, as was the quantification of neurites in N1E-115 cells. Primary antibodies used for immunoblotting: phospho-Ser144 PAK antibody (rabbit; Cell Signaling, #2606S, 1/1000), PAK1 antibody (mouse; Santa Cruz sc-166887, 1/1000), GFP antibody (rabbit; Torrey Pines Biolabs, #TP401, 1/3000).

Statistical analyses of phospho-PAK levels were made by non-parametric One-way ANOVA, Kruskal-Wallis test and Dunns’ post-test. Asterisks indicate datasets significantly different from WT (\*\*\*p<0.001). Statistical analyses of neurite outgrowth were made by One-way ANOVA, Dunnett test (\*\*\*p<0.001) (Figure 1).

**Functional study on splice site variant c.6244-2A>G**

Primers were designed to span exon 43 (ACACGAGAGAAGGTTGCACA) and exon 45 (ATGCACATGACTTCCACAGC) of TRIO transcript NM\_007118.4. cDNA was synthesised using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). PCR was performed using GoTaq G2 DNA polymerase kit (Promega, USA). Gel was prepared at 3% agarose in Nancy-520 DNA gel stain (Sigma, USA). Gel picture was documented using Invitrogen iBirght Imager (Thermo Fisher Scientific, USA). RT‐PCR products were purified by GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) and bidirectional Sanger sequencing was carried out by SourceBioscience. PCR experiments were repeated at least twice for reproducibility.

**Results**

A total number of 25 individuals with a pathogenic or likely pathogenic *TRIO* variant (reference sequence: NM\_007118.4) have been recruited into the current cohort, 9 of which had a variant in the spectrin domain (indviduals 1-9, Table 1), 7 had a variant in or around the GEFD1 and GEFD2 domains (individuals 10-16, Table 2), 8 had truncating variants and 1 participant had an in-frame intragenic deletion (individuals 17-25, Table 3). Of the 25 individuals, 13 were females and 12 males with the age range being 22 months to 46 years.

Patients 14 and 15 carried a variant that was classified as variant of uncertain significance (VUS) (p.Asn1465Ser). Of note, these individuals were unrelated. We therefore performed functional studies on this variant to provide evidence for pathogenicity. Since this mutation is located in the RAC-activating GEFD1 domain of TRIO, we monitored the effect of the N1465S variant on the phosphorylation levels of PAK1, a target of RAC1, which serves as a read-out for RAC1 activation, as described previously (Barbosa et al, 2020). As shown in Figure 1A-B, the N1465S variant impaired the ability of TRIO to activate RAC1, as phosphoPAK levels were significantly reduced, similarly to the GEFdead control. To confirm this effect, we used the N1E-115 neuroblastoma cell line, in which TRIO expression leads to enhanced neurite outgrowth, in a RAC1-dependent manner. As shown in Figure 1C, the N1465S variant was impaired in inducing neurite outgrowth. These two data allowed us to reclassify the p.Asn1465Ser VUS into the likely pathogenic category.

In addition, the pathogenicity of the variant c.6244-2A>G identified in Patient 23 was confirmed by splicing analysis. This has shown aberrant alternative acceptor site activation causing a 26 base pair loss and subsequent frame shift confirming pathogenicity. This result was confirmed by Sanger sequencing (see supplementary Figure 1).

***Phenotypic characteristics of patients with a missense variant in the Spectrin domain***

All 9 cases who possessed missense variants in the Spectrin domain had moderate to severe delay in their development, with a recognisable facial gestalt including macrocephaly (*Table 1 and Figure 2*).

*Neurodevelopmental and neurological phenotype*

All 9 patients have significant developmental delay ranging from moderate to severe with 4 of 9 (44.4%) cases not walking at the time of their assessment and 4 individuals being non verbal (three individuals were over 5 years of age at the time of their assessment and one patient was 3 years old). 4 out of 9 (44.4%) cases developed seizures and these included nocturnal seizures, absence seizures and myoclonus dystonia.

*Behavioural phenotype*

Behaviour issues were clearly observed, with 3 of the 9 (33.3%) cases having stereotypies, 3 of 9 (33.3%) agression, 3 of 9 (33.3%) obsessive-compulsive (OCD) traits and 5 of 9 (55.5%) cases with a missense variant had poor attention. Of note, 2 cases had attention deficit hyperactivity disorder (ADHD), one had hyperactivity and two had autism.

*Gastrointestinal, skeletal, dental and other features*

Infantile feeding difficulties were observed in 5 of 9 (55.5%) cases and 2 patients required tube feeding. 4 of the 9 (44.4%) cases had constipation, and intestinal malrotation, increased appetite and eosiniphilic oesophagitis was described in a single case. Of note, 5 of the 9 cases had growth retardation. Skeletal features also occurred in single cases, and included kyphosis, scoliosis and high arched feet. Short, tapering fingers were observed in two cases and long toes in a single case. Dental overcrowding was reported in 2 cases, while delayed dental eruption and missing teeth were described in one case. One participant had cardiac problems with bicuspid aortic valve, prominence of the aortic root and mild aortic regurgitation. None of the cases had genitourinary problems apart from possible neuropathic bladder in one case. An intriguing finding was the presence of brain malformations in patients 3, 4 and 6, including Arnold-Chiari malformation, thin corpus callosum and delayed myelin maturation (Table 1).

*Facial characteristics*

Characteristic facial features were recognised in the seven cases where this information was available and included high forehead, frontal bossing, receding hairline, frontal baldness, low-set ears, broad nasal tip, thin upper lip and synophrys (Figure 2).

***Phenotypic characteristics of patients with a missense variant in the GEFD1 and GEFD2 domains***

*Neurodevelopmental and neurological phenotype*

All of the 7 inviduals with a likely pathogenic or pathogenic variant in the GEFD1 (6 cases) or the GEFD2 domain (1 case) had mild to moderate developmental delay with microcephaly, except for one case (patient 13) who was described to have macrocephaly. Only patient 10 was reported not to have any words at 3.5 years of age. Patient 10 had myoclonic seizures and patient 11 had hypotonia (Table 2).

*Behavioural/psychiatric phenotype*

Information regarding behavioural issues was provided in 4 out of the 7 participants and 2 of 4 (50%) had stereotypies, 3 of 4 (75%) exhibited aggressive behaviour, 3 of 4 (75%) were descibed as having poor attention and 2 of 4 (50%) had OCD traits. One of the 7 cases had autism (patient 11) and 1 of 7 (patient 15) had hyperactivity (Table 2).

*Gastrointestinal, skeletal, dental and other features*

2 of 4 (50%) individuals were reported to have infantile feeding difficulties with one of the two cases requiring tube feeding. In addition, 3 of 4 (75%) cases had constipation and 3 of 4 (75%) cases had growth retardation.

Skeletal features were observed in this group of patients with 2 of 4 (50%) having scoliosis, 3 of 4 (75%) cases having short tapering fingers and 2 of 4 (50%) 2-3 toe syndactyly. Pectus excavatum, pes planus and hypermobility were observed in single cases. Dental overcrowding was seen in 1 of 4 cases (patient 10) and the same individual (patient 10) had delayed dental eruption (Table 2).

*Brain malformations*

Structural brain abnormalities were described in 2 cases and included hypoplasia or agenesis of corpus callosum in one participant (patient 10). Patient 10 also had dysmorphic lateral ventricles and dysmorphic basal ganglia. In addition, one patient (patient 16) had gliotic scarring in the right middle cerebral artery (MCA) and duplication of the inferior branch of the right MCA and a further individual (patient 13) had syrinx of the spine (Table 2).

*Facial characteristics*

Photographs of 5 of the 7 cases were made available, which revealed a recognisabe facial gestalt including microcephaly with hypertelorism, upslanting palpebral fissures, synophrys; long tubular nose with a bulbous nasal tip and thin upper lip (Figure 3).

***Phenotypic characteristics of patients with truncating variants and the intragenic deletion in the TRIO gene***

*Neurodevelopmental and neurological phenotype*

9 cases with a truncating variant (TV) or a deletion in the *TRIO* gene were identified in the current study. Of note, Patient 19 has already been inlcuded in a study by Kolbjer et al (Kolbjer et al., 2021). 7 of the 9 cases had mild to moderate developmental delay and one individual (patient 19) with the intragenic deletion had severe delay with all but one (patient 25) participants having microcephaly. Interestingly, the early development of two patients (patients 22 and 24) with truncating variants was within the normal range. Two cases (patients 19 and 25) with a TV had seizures and another participant (patient 20) had hypotonia.

The patient 19 with in-frame intragenic deletion had severe delay and never walked or talked. He was also described as having intractable seizures (Table 3).

*Behavioural/psychiatric phenotype*

Of the 8 individuals with a truncating variant, 2 (25%) had stereotypies, 3 (37%%) had agression, 7 (87%) had attention deficit and 3 (37%) had OCD traits. In addition, 2 of 8 (25%) had autism and one (12%) had hyperactivity. One patient (patient 22) had a diagnosis of bipolar disorder and another individual (patient 18) had mood swings and paroxysmal bursts of laughter. Patient 25 presented with self-mutilating behaviours since a very young age. Of note, one patient (patient 21) had anxiety and sleep paralysis/ REM sleep disorder associated with hallucinations from teenage years requiring sertaline medication (Table 3).

*Gastrointestinal, skeletal, dental and other features*

Infantile feeding problems were present in 7 of the 9 (78%) cases including the patient with the intragenic deletion with only the latter and another patient requiring tube feeding. Constipation was recorded in 2 of 9 (22%) cases and 5 out of 9 (55%) cases were described as having growth retardation. Skeletal features did not appear to be common in this group of patients, with short, tapering fingers described in two cases with TV; one of these individuals (patient 20) also had a short fifth toe and short nails. Dental crowding was described in one individual (patient 22) and delayed dental eruption was seen in two cases (patients 19 and 22). Of note, large incisors were detected in 2 of 9 cases (patients 17 and 19); and abnormality of the dental enamel (patient 17) and small teeth (patient 20) in single cases. In terms of cardiac features, one patient (patient 20) with a TV had atrial septal defect, patient 21 had tetralogy of Fallot and another case had suspected arrhythmia (patient 24). One patient (patient 20) had hypothyroidism requiring thyroxine and developed profound anaemia necessitating blood transfusion (Table 3).

*Brain abnormalities*

Structural brain malformation were seen in four cases (patients 18, 19, 23 and 24) and these included ventriculomegaly, lissencephaly,global cerebral atrophy, secondary agenesis of corpus callosum and progressive leucoencephalopathy on the MRI brain scan (Table 3).

*Facial characteristics*

Photographs were provided for 6 of the 9 cases and clinical features appeared to resemble those seen in individuals with missense variants in the GEFD1/2 domain. These included microcephaly, upslanting palpebral fissures, long, tubular nose with a bulbous nasal tip and thin upper lip (Figure 4).

**Discussion**

The *TRIO* gene encodes a protein that exchanges GDP to GTP on Rho GTPases and has a role in the remodeling of the cytoskeleton, hence impacting on cell migration and growth (Schmidt & Debant, 2014).

Pathogenic variants in the *TRIO* gene have been linked to neurodevelopmental problems including developmental delay, learning difficulties, behaviour issues such as ADHD and autism. Ba et al (Ba et al., 2016) described 4 individuals, 1 with an intragenic *de novo* deletion of *TRIO*, and 3 with truncation variants. All cases were described to have mild intellectual disability along with behaviour problems, comprising aggressive behaviour, hyperactivity and autistic features. In 2016, Pengelly et al (Pengelly et al., 2016) reported three members (proband, father and paternal uncle) of the same family with the same frameshift variant exhibiting similar facial features, mild learning difficulties, microcephaly, skeletal and dental features, as well as an additional three cases with missense variants (one in the Spectrin and two in the GEFD1 domains). The two cases with missense variants in the GEFD1 domain had microcephaly and the third patient with the spectrin variant had macrocephaly. More recently, Barbosa et al (Barbosa et al., 2020) reported a cohort of 24 individuals (which included cases reported by Pengelly et al.) with pathogenic nonsense and missense variants and reported a distinct phenotype and genotype effect between sequence changes in different domains. All individuals presented in that study had a degree of neurodevelopmental delay and behavioural issues, but those with missense variants in the spectrin domain were found to have severe intellectual disability and macrocephaly, while those with missense variants in the GEFD1 domain had a milder phenotype with microcephaly. Truncating variants were scattered across the gene and were associated with variable degree of neurodevelopmental problems. Kloth et al (Kloth et al., 2021) also reported two cases with variants in the spectrin domain demonstrating macrocephaly, characteristic facial features and moderate to severe developmental delay. One of the cases had stereoptypies and seizures and both cases presented with growth problems. Skeletal abnormalities and structural brain malformations were observed in one of the cases. In addition, Schultz-Rogers et al(Schultz-Rogers et al., 2020) reported two cases with truncating variants who had developmental delay, one of them had macrocephaly and the other had microcephaly. Interestingly patient 2 presented with cutis aplasia. One of the patients had steretotypies, agression, poor attention span and seizures.

Here we provide detailed description of clinical features of an additional 25 individuals, 9 of whom have a missense variant in the Spectrin domain, 6 cases with a missense variant in the GEFD1 domain, 1 case with a missense variant in the GEFD2 domain, 8 patients with truncating variants and 1 individual with a in-frame intragenic deletion (Figure 5).

Comparing the 25 cases presented here with those reported previously (see Table 4), there appears to be a recognisable split between clinical features of individuals with a missense variant in the Spectrin domain and those with a missense variant in the GEFD1 or GEFD2 domains, truncating variants or deletions.

*Missense variants in the spectrin domain*

All 9 patients presented here were delayed in the acquisition of their developmental milestones ranging from moderate to severe, which is in keeping with findings from previous studies. Short stature was described in three cases here and growth retardation in 5 of the 9 cases, in addition to one case that was published by Pengelly et al (Pengelly et al., 2016). All cases, where measurements of the head circumference were available, had absolute – or in a few cases relative - macrocephaly, which is identical to *TRIO* cases in other case series with this finding. Neurobehavioural phenotypes were previously described in the Pengelly et al (2016) paper and included stereotypies, agression and poor attention span. Barbosa et al (Barbosa et al., 2020) also described an overall incidence of 36% for agression, 27% for stereotypies, 45% OCD traits, 31% autistic traits and 70% had poor attention in the *TRIO* cohort. In our spectrin cohort, the rate of stereotypies was 33.3 % (3/9), agression was 33.3% (3/9), poor attention was 55.5% (5/9) and OCD traits was 43% (3/7). In addition, hyperactivity, autism and ADHD were described as in previous papers. Seizures occurred in 4 of 9 cases (44.4%) which is similar to those seen in the Barbosa paper (33.3 %). Infantile feeding difficulties were delineated in the spectrin case of the Pengelly paper and found in 55.5% (5/9) in the cases here and constipation was seen in 44.4% (4/9) of this patient cohort. Facial features were described in previous papers and adds to the evidence for a recognisable facial gestalt comprising high forehead, frontal bossing, receding hairline, frontal baldness, low-set ears, broad nasal tip, thin upper lip and synophrys. The presence of brain malfomations has been alluded to in the Barbosa et al., 2020 study and described here in detail, including Arnold-Chiari malformation, thin corpus callosum and delayed myelin maturation.

*Missense variants in GEFD1 and GEFD2, intagenic deletion and truncating variants present with a similar phenotype*

Of the 7 cases with GEFD1/2 variants, 5 had microcephaly (or relative microcephaly) and all had mild to moderate developmental delay, which was consistent with findings from other studies athough one patient (Patient 13) was reported to have macrocephaly with no actual measurement of the head circumference. Interestingly, patients with TVs and the deletion also had microcephaly with no reported cases with macrocephaly. All of the TV cases had mild to moderate delay versus the one deletion case who had severe learning issues. In contrast, there were two TV cases in the Barbosa et al paper who had severe delays. None of the cases with GEFD1 or GEFD2 missense variants had short stature which was consistent across the case series in previous studies, however 3 of 13 (23 %) of all reported TV and deletion cases reported so far had short stature.

Neurobehavioural phenotypes were described in the current cohort (inlcuding patients with GEFD1/2 variants, TVs and the intragenic deletion) with stereotypies (4/13, 33.3%), agression (6/13, 46%), poor attention (10/13, 77%), OCD traits (5/13, 38%) and seizures (4/13, 31%) being described. These features were also delineated in the Barbosa study, although only an overall figure was presented for the *TRIO* cohort there. Of note, neurobehavioural phenotypes appeared to be slightly less common in the TV group (including the intragenic deletion) with the exception of poor attention that was seen in 7 of the 9 (78%) cases (Table 4). Infantile feeding difficulties (9/13, 61%), tube feeding (3/13, 23%) and constipation (5/13, 38% were observed in the GEFD1/2 and the TV cohort as well, with similar ratios to previous reports (Ba et al., 2016; Pengelly et al., 2016). Facial features were similar in all of these cases and included microcephaly with hypertelorism, upslanting palpebral fissures, synophrys, long tubular nose with a bulbous nasal tip and thin upper lip.

The current study highlights possible psychiatric complications such as anxiety, sleep paralysis/ REM sleep disorder associated with hallucinations, bipolar disorder and mood swings with paroxysmal bursts of laughter that are all reported in single cases.

In addition, we report brain abnormalities, namely hypoplastic or agenesis of corpus callosum, dysmorphic lateral ventricles, dysmorphic basal ganglia, global cerebral atrophy, lissencephaly, ventriculomegaly, progressive leukoencephalopathy, gliotic scarring in the right middle cerebral artery (MCA) and duplication of the inferior branch of the right MCA.

Patient 19 has an intragenic deletion of *TRIO* and 8 patients had truncating variants suggesting that haploinsufficiency of the *TRIO* gene through a loss of function mechanism is the likely molecular cause of the phenotypes observed in these cases. However, we do not know whether the frameshift variants result in truncated products of different sizes or nonsense-mediated decay of the aberrant transcripts. Similarities of clinical features observed in cases with variants in the GEFD1/GEFD2 and TVs are evident and could be explained by the effect of the GEFD1 variants on RAC1 leading to reduced activity of the TRIO protein and a loss-of-function mechanism as shown by Barbosa et al (2020).

The current study further supports the finding that missense variants in the spectrin domain cause more significant developmental delay with prominent neurological and behaviour features including seizures, stereotypies, autism, OCD and hyperactivity, while frameshift and GEFD1/2 variants cause a milder phenotype with mild delay but also with significant neurological and behaviour involvement. Difference in the phenotype was not observed in gastrointestinal, dental and skeletal phenotypes.

In view of these findings, we recommend neurodevelopmental, dental and spine surveillance for individuals with *TRIO* gene variants, with brain MRI performed at their initial assessment. In addition, gastrointestinal, psychiatric and behavioural assessments are recommended.

Further studies are required to determine the possibility of long term complications related to *TRIO* and to uncover the significance of the structural brain malformations and the psychiatric issues.

In summary, this case series brings the number of reported *TRIO* cases to 57 patients with either a variant in spectrin and a likely gain-of-function mechanism or a variant in the GEFD1/2 domain, truncating variant or a deletion likely leading to a loss-of-function of the TRIO protein, resulting in distinguishable clinical features specific to the effect of the variant on the gene function. This current cohort further supports the contention raised by previous publications that missense variants in the Spectrin domain result in a distinct phenotype with recognisable features (macrocephaly, frontal bossing etc.) while truncating variants, deletions and missense variants in the GEFD1 and 2 domains are more likely to lead to a loss of protein function and charactersitic features that include microcephaly, long, tubular nose, bulbous nasal tip and thin upper lip amongst other features.

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**Author contribution statement**

Conceptualization: DB, AD, SS, GG; Data curation: GG, DH, AMCG, MPR, AC, MM, FV, DS, AC, E-LS, B-MA, ST, ESA, CMcW, PRW, MO’D, GA, AKB, PZ, LDM, AGML, OA-L, PB, HW, OR; Formal Analysis of the clinical data: GG, Analysis of functional data: AD, SS, HW, OR, DB; Investigation and interpretation of data: GG, HW, OR, DB, SS, AD; Methodology: GG, AD, SS, DB; Supervision: DB, AD, SS; Visualisation: GG, SS, AD, HAW, OR; Writing – original draft: GG; Writing – review & editing: DH, AMCG, MPR, AC, MM, FV, DS, AC, E-LS, B-MA, ST, ESA, CMcW, PRW, MO’D, GA, AKB, PZ, LDM, AGML, OA-L, HAW, OR, PB, AD, SS, DB.

# Legends to Figures

**Fig.1 TRIO-N1465S variant leads to** **decreased neurite outgrowth and impaired RAC1 activation. (A)** Quantification of the ratio of phospho-PAK1 levels over total PAK1 expression, measured from HEK293T cell lysates transfected with the indicated GFP-TRIO constructs. GEF-dead is a TRIO form mutated in its GEF domain and unable to activate RAC1. Data are presented as the mean ± SEM of at least four independent experiments. **(B)** Representative immunoblot of HEK293T cell lysates transfected with the indicated GFP-TRIO constructs and detected with an anti-GFP antibody. PAK1 phosphorylation and total levels are detected with PAK1 antibodies, recognising phosphorylated PAK on Ser144 or total PAK, respectively.

**(C)** Quantification of neurite outgrowth induced by WT or TRIO-N1465S variant in N1E-115 cells. Data are presented as n-fold change over WT TRIO, which was arbitrarily set to 1. Data are presented as the mean ± SEM of at least four independent experiments.

**Fig.2** Facial and limb features of individuals with likely pathogenic or pathogenic variant in the spectrin domain of the *TRIO* gene. Common features are high forehead, long nose with broad nasal tip, synophrys and low-set ears.

**Fig. 3** Facial and limb phenotypes of individuals with a missense variant in the GEFD1 or GEFD2 domain.

**Fig. 4** Facial and limb features of individuals with a truncating variant. Patient 19 possesses an in-frame intragenic deletion.

**Fig.5** TRIO domains map showing the position of variants described in this study.

Supplementary Figure1 Pathogenicity of the variant c.6244-2A>G identified in Patient 23 was confirmed by splicing analysis showing an aberrant alternative acceptor site activation causing a 26 base pair loss and subsequent frame shift. This result was confirmed by Sanger sequencing.

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