Risk pathways to autism in a cohort of children and adolescents with Tuberous Sclerosis Complex

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Abbreviations: ADI-R = Autism Diagnostic Interview–Revised; ADOS-2 = Autism Diagnostic Observation Schedule, second edition; AIC = Akaike information criterion; ASD

= Autism spectrum disorder; BIC = Bayesian information criterion; BPVS = British Picture Vocabulary Scale; EFA = Exploratory factor analysis; IQ = Intelligence Quotient; MSEL = Mullen Scales of Early Learning; mTOR = mammalian target of rapamycin; PKD1 = Polycystic Kidney Disease 1; PTEN = phosphatase and tensin homolog; RMSEA = root mean square error of approximation; SABIC = sample-size-adjusted Bayesian information criterion; SEGA = subependymal giant cell astrocytoma; TSC = Tuberous Sclerosis Complex; TS2000 = Tuberous Sclerosis 2000 Study; VABS = Vineland Adaptive Behaviour Scales extended survey parental interview; VABS-II = Vineland Adaptive Behaviour Scales, Second Edition; WASI-II = Wechsler Abbreviated Scale of Intelligence – Second Edition

Abstract

Background: Tuberous Sclerosis Complex (TSC) is a single gene disorder carrying high risk of autism spectrum disorder (ASD). Various neurological complications increase the risk of ASD but the way risk factors operate together is unclear. We aimed to explore risk pathways to ASD by modelling the interplay between genetic mutation (TSC1/TSC2), cortical tuber count, seizure type and severity. **Methods**: The Tuberous Sclerosis 2000 Study is a UK longitudinal study of the natural history of TSC. We recruited newly diagnosed children (*N*=125) and collected data on mutation, cortical tuber count (cranial MRI/CT), seizure history, and IQ. ASD and IQ assessments were carried out at 10-year follow up (*N*=86, *M* age=13.1 years). Structural equation modelling (SEM) was used to explore pathways that mediate between mutation and ASD symptoms. **Results**: Risk of ASD was high: 39.5% met research criteria for ASD and a further 41.9% showed autistic traits. SEM resulted in two indirect pathways, with cortical tuber count and occurrence/severity of epileptic spasms in infancy mediating between mutation and ASD (*mutation-tubers-spasms-ASD*, *B*=2.08, 95%*CI* 0.15–8.02; *mutation-spasms-ASD*, *B*=2.98, 95%*CI* 0.04–8.89). Concurrent seizures (*B*=3.08, 95%*CI* 0.42–6.18) and IQ (*B*=-117.10, 95%*CI* -183.57–-59.16) were also associated with ASD symptoms. **Conclusions**: There was significantly elevated risk of ASD and subclinical autistic traits. Tuber count and severity of spasms predicted ASD severity, suggesting that prevention/control of seizures in infancy may decrease severity of ASD symptoms. ASD was occasionally reported in the absence of overt seizures in infancy, so their causal role requires further investigation.

Keywords: Autism spectrum disorder; tuberous sclerosis; seizures; infantile spasms; longitudinal

General Scientific Summary: This study followed children and young people with the genetic disorder Tuberous Sclerosis Complex (TSC) for over 10 years and found that four out of every 10 children developed autism. More severe brain abnormalities and seizures in infancy increased the likelihood of autism; early control of seizures might reduce the severity of autism in children and young people with TSC.

INTRODUCTION

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterised by deficits in social interaction and communication, repetitive and stereotyped behaviours and interests, and sensory atypicalities, with prevalence of at least 1% (Lord, Elsabbagh, Baird, & Veenstra-Vanderweele, 2018). Despite substantial heritability estimates (64-91%; Tick, Bolton, Happe, Rutter, & Rijsdijk, 2016), heterogeneity makes characterisation of the genetic architecture of ASD challenging (Betancur, 2011) and complicates mapping of causal pathways. In several other diseases, research on single gene disorders associated with neuropsychiatric conditions has resulted in inroads into identifying underlying mechanisms. For example, study of familial cases with known genetic causes has benefited the study of Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (Sahin, 2012). With ASD, there is increasing evidence for convergence on common pathways, such as inherited and de novo genetic risk variants (identified in multiplex and simplex families respectively) converging on protein-protein interaction networks with relevance to cortical neurogenesis (Ruzzo et al., 2019). Single gene disorders have potential as models for ASD and may complement the use of infant siblings of children with ASD as a way of studying early development in ASD (Szatmari et al., 2016).

Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is a single gene disorder often diagnosed early in development, including prenatally (Davis, Peters, Krueger, & Sahin, 2015): 26-50% of individuals with TSC have ASD (Davis et al., 2015) and the symptom profile does not differ from non-syndromic ASD (Jeste et al., 2016). TSC is an autosomal dominant disorder, caused by mutation in the TSC1 or TSC2 gene, resulting in abnormal cellular differentiation, proliferation and migration that result in hamartomas in various organ systems (Curatolo,

Moavero, & de Vries, 2015). Together with Fragile X, neurofibromatosis type 1, and disorders associated with phosphatase and tensin homolog (PTEN) mutations, it signals through the mTOR pathway (de Vries, 2010), a key regulator of protein synthesis, suggesting that this may be one common pathway underlying ASD (Sahin, 2012).

TSC results in structural brain abnormalities at micro- and macroscopic levels. Macroscopic lesions include cortical tubers, radial migration lines, subepyndymal nodules, and subependymal giant cell astrocytomas (SEGAs), while white matter involvement results in disruption to structural brain networks (Davis et al., 2015; Im et al., 2016). Impaired microstructure in regions important for language have been documented in TSC and is most pronounced in those with TSC and ASD (Lewis et al., 2013). Measures of disrupted connectivity across the whole brain are correlated with tuber load, suggesting that tubers may act as a marker for diffuse changes (Im et al., 2016). Disturbance to connectivity may be a common feature of ASD that increases risk for epilepsy (which occurs at a rate of around 30% in ASD and 80-90% in TSC; Peng, Zhou, & Wang, 2021; Sahin, 2012). Importantly, electrophysiological perturbations associated with early onset epilepsy may also play a causal role in long-term changes including disrupted connectivity (Holmes, 2005).

TSC allows us to look at risk pathways to ASD in a disorder in which the genetic and neural architecture are increasingly understood, with the potential to identify candidates for common pathways to ASD. This study involves a well-characterised nationally-representative cohort of children and young people with TSC, the Tuberous Sclerosis 2000 Study, who have been followed for over 10 years. This allows exploration of the relative influence of risk factors known to be associated with ASD, including genetic mutation; cortical tubers; type, severity, age of onset, and course of seizures; and intellectual disability (Davis et al., 2015; Gupta et al., 2020). Specific aims are to: (i) estimate the prevalence of ASD and subthreshold autistic

difficulties; (ii) investigate tuber count and epilepsy characteristics as risk factors for ASD; and (iii) model the interplay between risk factors during development.

MATERIALS AND METHODS

Sample

The Tuberous Sclerosis 2000 Study (TS2000) is a UK population-based, prospective study of the natural history of TSC. Ethical approval and written informed consent was obtained at each phase. See Supplementary Section 1 and Yates et al. (2011) for full details. In Phase 1 (2001-2005), 125 participants met diagnostic criteria for TSC and completed study assessments (49.6% male, median age at recruitment=2.7 years, range=4 weeks–18 years). Participants were assessed by expert clinicians in a network of clinics covering the UK: a full medical history was obtained and physical examination carried out using a standardized protocol (Yates et al., 2011). During Phase 2 (2012-2015), participants completed assessments for ASD (*n*=86) and cognitive ability (n=88) (45% male, median age=12.5 years, range=7.8-26.9 years, 91.9% of the sample aged<18). Seizure history was updated and recent brain scans collected where possible.

Measures

Genetic mutation

Genotyping was carried out by two diagnostic laboratories providing TSC mutation testing in the UK (East Anglian Regional Genetics Laboratory, Cambridge and the Institute of Medical Genetics, Cardiff; Yates et al., 2011). All exons and flanking intronic sequences of the TSC1 and TSC2 genes were screened for point mutations. Samples were tested for whole exon deletions (including TSC2/PKD1) by multiplex ligation-dependent probe amplification

(MLPA; MRC-Holland, Amsterdam, The Netherlands). The causal mutation was determined for 96 children (TSC1 *n*=19; TSC2 *n*=77). In 7 children a pathogenic mutation could not be identified and in 22 children genetic testing was not carried out.

Cortical tuber count

Cranial CT or MRI was carried out as part of routine clinical care in the majority of cases and scans were reviewed during Phase 1 (*n*=86). During Phase 2, more recent scans (*n*=41) and new scans (*n*=23) were obtained where possible, increasing the total to *n*=109. Scans were reviewed and rated without knowledge of other clinical details by JNPH (Phase 1) and FS (Phase 2) using a pre-specified coding system that recorded the number and lobar location of cortical tubers and the presence of subependymal nodules. The inter-rater reliability of this procedure is acceptable (Bolton, Park, Higgins, Griffiths, & Pickles, 2002). Where more than one scan was available, the most recent scan or the scan of highest quality was used. Tuber count was summated for each major lobe of the brain (Table 1 and Supplementary Section 2).

Table 1. Description of the sample by genetic mutation status

	N (%) of sample with data	Overall	TSC1 mutation (n=19)	TSC2 mutation (n=77)	No mutation identified (n=7)	Not tested (n=22)	χ^2(df) or *F*(df)	Posthoc test
Sex, M:F	125 (100%)	62:63	14:5	32:45	5:2	11:11	7.63 (3), p=.055	
Age of autism assessment in years;months, mean (SD) [range]	86 (68.8%)	13.1 (3.8) [7.8-26.9]	13.8 (4.1) [9.3-25.8]	12.7 (3.7) [7.8-26.9]	11.6 (4.5) [8.0-17.8]	14.1 (3.8) [9.9-24.6]	0.94 (3,82), p=.423	
Cortical tuber count for all major lobes, mean (SD) [range]	109 (87.2%)	19.31 (14.42) [0-68]	6.13 (6.40) [0-22]	22.01 (14.54) [0-68]	27.86 (14.92) [7-48]	16.89 (12.16) [0-43]	**6.96 (3,105), p<.001**	TSC1 < TSC2 / NMI / no testing
Seizures ever, n (%)	125 (100%)	114 (91.2%)	16 (84.2%)	72 (93.5%)	7 (100.0%)	19 (86.4%)	2.98 (3), p=.422	
Epileptic spasms ever, n (%)	121 (96.8%)	63[A] (52.1%)	6 (31.3%)	41 (53.2%)	3 (42.9%)	13 (61.9%)	4.58 (3), p=.209	
Other seizure types, n (%)	121 (96.8%)	106 (87.6%)	16 (84.2%)	66 (89.2%)	7 (100.0%)	17 (77.2%)	2.22 (3), p=.557	
Age of seizure onset in months, mean (SD) [range]	109 (87.2%)	11.96 (17.96) [0-122]	30.88 (32.69) [3-122]	9.05 (10.55) [0-60]	14.43 (25.51) [1-72]	5.18 (4.06) [0.75-18.00]	**9.07 (3,105), p<.001**	TSC2 / NMI < TSC1
Seizure severity year 1, mean (SD) [range]	125 (100.0%)	0.00 (0.98) [-1.16–1.58]	-0.70 (0.85) [-1.16–2.43]	0.09 (1.00) [-1.16–1.58]	0.10 (0.98) [-1.16–1.28]	0.25 (0.74) [-1.16–1.27]	**4.25 (3,121), p=.007**	TSC1 < TSC2 / no testing
Seizure severity year 2, mean (SD) [range]	125 (100.0%)	0.00 (0.98) [-1.30–1.22]	-0.41 (1.08) [-1.30–1.22]	0.04 (0.98) [-1.30–1.22]	0.11 (0.78) [-1.30–1.07]	0.19 (0.89) [-1.30–2.51]	1.49 (3,121), p=.222	
Epileptic spasm severity year 1, mean (SD) [range]	120 (96.0%)	0.00 (0.98) [-0.82–1.91]	-0.63 (0.57) [-0.82–1.11]	0.09 (1.01) [-0.82–1.91]	-0.09 (1.18) [-0.82–1.19]	0.28 (0.93) [-0.82–1.46]	**3.67 (3,116), p=.014**	TSC1 < TSC2 / no testing
Epileptic spasm severity year 2, mean (SD) [range]	120 (96.0%)	0.00 (0.98) [-0.59–2.07]	-0.28 (0.77) [-0.59–1.90]	0.05 (1.03) [-0.59–2.07]	-0.14 (1.08) [-0.59–2.07]	0.10 (1.03) [-0.59– 1.98]	0.67 (3,116), p=.574	
Other seizures severity year 1, mean (SD) [range]	119 (95.2%)	0.00 (0.98) [-0.84–2.00]	-0.36 (0.84) [-0.84–1.46]	0.03 (0.99) [-0.84–2.00]	0.73 (0.88) [-0.84–2.67]	-0.06 (0.99) [-0.84–1.57]	2.22 (3,115), p=.089	TSC1 < NMI

Other seizures severity year 2, mean (SD) [range]	119 (95.2%)	0.00 (0.97) [-1.19–1.46]	-0.45 (0.96) [-1.19–1.21]	0.06 (0.98) [-1.19–1.46]	0.29 (0.83) [-1.19–1.20]	0.07 (0.89) [-1.19–1.21]	1.69 (3,115), p=.172
Seizure severity age 7+ years, mean (SD) [range]	94 (75.2%)	0.00 (0.98) [-1.19–1.79]	0.17 (1.11) [-1.19–1.79]	0.01 (0.98) [-1.19–1.70]	0.04 (1.04) [-1.19–1.39]	-0.23 (0.87) [-1.19–0.90]	0.40 (3,90), p=.752
ASD							
ADI-R diagnostic algorithm score, mean (SD) [range]	85 (68.0%)	30.40 (16.28) [1–60]	27.93 (16.53) [9-54]	30.00 (15.51) [1-60]	34.04 (22.51) [5-55]	33.49 (18.31) [5-57]	0.35 (3,81), p=.788
ADI-R current behaviour algorithm score, mean (SD) [range]	85 (68.0%)	20.54 (14.90) [0–55]	18.22 (12.76) [5-42]	19.39 (14.20) [0-48]	25.32 (21.86) [2-49]	25.93 (17.54) [3-55]	0.97 (3,81), p=.410
ADOS-2 Comparison Score[B], mean (SD) [range]	80 (64.0%)	4.53 (3.01) [0-10]	3.98 (2.81) [0-9]	4.42 (3.00) [0-10]	4.44 (3.60) [0-7]	5.59 (3.22) [0-10]	0.72 (3,76), p=.542
ASD factor score, mean (SD) [range]	86 (68.8%)	0.00 (0.97) [-1.60-2.01]	-0.17 (0.87) [-1.16-1.42]	-0.05 (0.93) [-1.60-1.67]	0.28 (1.42) [-1.44-1.73]	0.30 (1.11) [-1.44-2.01]	0.76 (3,82), p=.523
ASD research classification	86 (68.8%)						
No ASD		16 (18.6%)	3 (18.8%)	10 (19.2%)	1 (25.0%)	2 (14.3%)	4.43 (9), p=.961
Broad spectrum		11 (12.8%)	3 (18.8%)	7 (13.5%)	0 (0.0%)	1 (7.1%)	
Probable ASD		25 (29.1%)	6 (37.5%)	14 (26.9%)	1 (25.0%)	4 (28.6%)	
ASD		34 (39.5%)	4 (25.0%)	21 (40.4%)	2 (50.0%)	7 (50.0%)	
Estimated IQ, mean (SD) [range]	124 (99.2%)	69.57 (20.80) [29-119]	77.68 (23.33) [38-119]	68.21 (19.00) [29-112]	66.57 (23.31) [42-101]	68.23 (23.50) [40-118]	1.16 (3,120), p=.330

[A] n=4 had spasms only, n=59 had both spasms and other seizure types.

[B] Number completing each module: Module 1= 14, Module 2=7, Module 3=40, Module 4=19.

Epilepsy

Using information derived from a specially devised interview schedule, seizure diary, and medical records, different features of epilepsy history were combined to generate a seizure severity score using the E-Chess (Humphrey et al., 2014). Exploratory factor analysis resulted in the following features loading onto one factor: (i) number of seizure types; (ii) duration of seizures; (iii) seizure frequency at most severe; (iv) number of anti-epileptic drugs used; and (v) response to treatment. Using confirmatory factor analysis, seizure severity factor scores were generated for three periods: first year of life (*Year 1*; *n*=120); second year of life (*Year 2*; *n*=120); and three months prior to Phase 2 assessment (*Age 7+*; *n*=94). Two additional scores were produced for the first two years of life: (i) *spasms severity*, which included all features for spasms (excluding number of seizure types); and (ii) *other seizures severity*, which included features for other seizure types (including number of seizure types other than spasms). A score for *other seizures severity ever* was calculated from information at all study points and used to dichotomise the sample into *no-to-low-severity* and *medium-to-high-severity other seizures* (mean severity difference, *d*=2.46). See Supplementary Section 3 for more detail about epilepsy scores.

Autism spectrum disorder and autistic traits

Assessment for ASD was carried out for 86 participants during Phase 2 using the Autism Diagnostic Interview–Revised (ADI-R) and the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2, module appropriate to age and language level; comparison scores reported). Different trained interviewers carried out the ADI-R and ADOS-2 for each child, blind to other results from the child. Interviewers regularly participated in ADOS and ADI-R reliability meetings (led by certified ADOS/ADI-R trainers) to maintain research reliability. ADI-R and ADOS-2 scores were used categorically to estimate diagnosis. Additionally, a

latent ASD factor score was generated using confirmatory factor analysis, using the ADI-R diagnostic and current behaviour algorithms, and ADOS-2 comparison score. This factor score was used as a continuous measure of ASD risk. See Supplementary Section 4 for more details about ASD classification and scores.

Estimated IQ

Following previous work, IQ was estimated for 124 participants (Tye et al., 2020). In Phase 1, intellectual ability was assessed using the Mullen Scales of Early Learning (MSEL) or Vineland Adaptive Behaviour Scales extended survey parental interview (VABS). In Phase 2, participants were administered the Wechsler Abbreviated Scale of Intelligence – Second Edition (WASI-II) or the British Picture Vocabulary Scale (BPVS), and/or the Vineland Adaptive Behaviour Scales, Second Edition (VABS-II). Data were available for 121 children in Phase 1 and 88 in Phase 2. A best-estimate IQ variable was created by using the WASI-II score if available, or alternatively the MSEL, VABS-II, or VABS, in that order of priority for 124 participants (Supplementary Section 5).

Data analysis

Descriptive statistics for the Phase 2 sample were compared to the baseline sample using the binomial test or one-sample *t*-test. Differences in demographic variables, risk factors, and outcomes by mutation status were tested using chi-square or Analysis of Variance (ANOVA). Spearman's rho was used to assess correlations. Student's *t*-test was used to explore the effect of dichotomous variables on ASD factor score, and ANOVA to test the relative role of various epilepsy factors while controlling for factors such as gender and IQ. Structural equation modelling (SEM) was carried out using Mplus (Version 7.31, Los Angeles, CA: Muthén & Muthén; 2015), using full information maximum likelihood estimation with robust standard errors to enable the inclusion of cases with missing data. Bootstrapping (1000

resamples) was used to estimate standard errors and 95% confidence intervals used to determine the significance of path coefficients; this approach is robust to small sample size and non-normality of data. We hypothesized that the risk for ASD would be mediated by tuber count and epilepsy, as has been demonstrated for intellectual impairment in this cohort, and this was used to guide specification of the SEM (Bolton et al., 2015; Tye et al., 2020).

Transparency and openness

Readers seeking access to these data should contact Dr Fiona McEwen or Dr Charlotte Tye, King's College London (fiona.mcewen@kcl.ac.uk or charlotte.tye@kcl.ac.uk). Access to restricted and fully anonymized data will be granted to named individuals in accordance with research governance procedures governing the reuse of data. Specifically, requestors must complete a study data access request form with details of proposed data usage and a formal data sharing agreement. This may require additional ethical approval depending on the nature of the request. Code is available at: https://osf.io/d2pk7/. All materials and methods are described and cited in the main manuscript or supplementary materials. The study is reported according to the STROBE Statement (von Elm et al., 2008). This study utilizes data from a longitudinal study initiated in the year 2000 that was not preregistered but the protocol is available on request (fiona.mcewen@kcl.ac.uk).

RESULTS

Sample

The baseline sample comprised *N*=125 children recruited in Phase 1; children who completed Phase 2 ASD assessments (*n*=86) were representative of the baseline sample across all demographic measures and risk factors, other than median age of seizure onset being slightly

higher (6.00 vs. 5.00 months, *p*=.002; Supplementary Section 1). Characteristics of the sample by genetic status are presented in Table 1. The SEM includes all 125 participants; where analyses are based on a subset with available data, *N* is indicated.

Cortical tuber count

Tuber count data were available for 109 (87.2%) children. The mean tuber count was 19.31 (SD=14.42) across the whole brain. Tuber count was greatest for the frontal lobe and lower for the temporal, parietal, and occipital lobes. Cerebellar tubers were rare, occurring in 8 (1.8%) individuals. See Supplementary Section 2.

Seizure history

The majority (*n*=114, 91.2%) of participants had a history of seizures, 52.1% of whom had epileptic spasms in infancy. Median age of onset was 5 months, and onset in those with spasms was earlier than those with other seizure types (median 4.0 vs. 12.5 months, Mann-Whitney *U*=-3.47, p=.001). All those with spasms had onset <24 months; spasms were more common in the first (*n*=52) than second year (*n*=33). Spasms co-occurred with other seizure types in 93.7% of cases. Seizure data at age 7+ years were available for 78 participants with ASD data and an additional 15 who did not complete ASD assessments. Around half (52.7%) had seizures in that period, which had continued for more than 6 months in most cases (95.9%). Exploratory factor analysis (EFA) showed that models where spasms and other seizure types loaded on separate factors fitted the data better than those that collapsed them onto one factor. See Supplementary Section 3.

Autism Spectrum Disorder

Thirty-four children and young people (39.5%) met both ADI-R and ADOS-2 criteria for ASD/autism and were classified as *ASD*. A further 25 (29.1%) met either ADI-R or ADOS-2

criteria and were classified as *Probable ASD*, and 11 (12.8%) met ADI-R Autism Genetic Resource Exchange criteria (AutismSpeaks, 2021) for broad spectrum but did not meet ADOS-2 criteria, so were classified as *Broad Spectrum*. Sixteen (18.6%) were below cut-off on both ADI-R and ADOS-2 and were classified as *No ASD*. While the ASD latent factor score appeared to have a bimodal distribution (Figure 1), this seemed to be driven by a small number of individuals and there was not strong evidence for bimodality (Bimodality Coefficient=.52; Supplementary Section 4.5, Figure S11).

Predictors of ASD severity

Total tuber count was correlated with ASD factor score (rho=.27, p=.017), as was tuber count for frontal (rho=.27, p=.016) and temporal (rho=.25, p=.025) lobes (n=80). However, presence of tubers in specific lobes was not associated with ASD when controlling for total tuber count (Supplementary Section 4.6 and Table S4). Spasms, status epilepticus, and medium-to-high-severity other seizure types were associated with higher ASD risk and different seizure types had an additive effect (Figure 2, Supplementary Section 4.7 and Figures S13-S16). After correcting for IQ, only spasms were associated with ASD factor score. Seven individuals had onset of seizures after the second year of life, four of whom had a best estimate research diagnosis of ASD or probable ASD.

Structural Equation Models (SEM)

Bivariate associations between variables were used to guide specification of the SEM (Table 1 and Supplementary Section 6). An SEM was specified with mutation (TSC1 vs. TSC2), cortical tuber count, spasms severity and other seizures severity, estimated IQ, and ASD. In line with results of EFA (Supplementary Section 3), the full model included spasms and other seizures in the first and second year of life as separate variables. A series of models were

specified, including more parsimonious nested models that collapsed seizure variables onto latent factors, and one that added age of seizure onset (Supplementary Section 7, Figure S18). All possible paths were included in the models, including those that were not statistically significant; it is thus a relatively conservative approach that avoids potentially inflating some path coefficients by removing paths of small effect size.

Fit statistics for the five models are presented in Table 2. The full model (1a) provided a satisfactory fit to the data, though the nested model (1b) showed an improvement in absolute fit statistics (e.g., RMSEA=0.056) and comparative fit statistics (AIC, BIC, and SABIC, model 1b<1a). Thus, collapsing year 1 and 2 seizure variables onto latent factors improved the model fit.

Table 2. Fit statistics for Structural Equation Models

Model		N sample statistics	Chi-square (df)	p	RMSEA[A] (90% CI)	CFI	SRMR[B]	AIC[C]	BIC[C]	SABIC[C]
1a	Full mediation model with spasms and other seizure types separately for year 1 and 2	66	28.68 (18)	.052	0.069 (0.000-0.114)	0.974	0.051	4585.11	4751.98	4565.41
1b	Mediation model with year 1+2 latent factors for spasms and other seizures	66	37.43 (27)	.087	0.056 (0.000-0.095)	0.974	0.050	4574.51	4715.92	4557.81
2	Mediation model as 1b but adding age of seizure onset	78	46.15 (31)	.039	0.063 (0.014-0.098)	0.967	0.043	5467.47	5634.34	5447.77
3a	Mediation model with all seizure types together but separately for year 1 and 2	45	23.07 (12)	.027	0.086 (0.028-0.138)	0.970	0.035	3947.85	4066.64	3933.83
3b	Mediation model with year 1+2 latent factor for all seizure types	45	36.47 (16)	.003	0.101 (0.058-0.145)	0.944	0.049	3951.60	4059.07	3938.91

AIC = Akaike information criterion; BIC = Bayesian information criterion; CFI = comparative fit index; RMSEA = root mean square error of approximation; SABIC = sample-size-adjusted BIC; SRMR = standardized root mean squared residual.

[A] The lower value of the 90% confidence interval should include or be very near zero (or no worse than 0.05) and the upper value should not be very large, i.e., less than .08; RMSEA penalises greater complexity.

[B] SRMR does not penalise for model complexity.

[C] AIC and BIC are comparative fit indices, lower values indicate better fitting models; penalise complexity; can only be used to compare nested models with same observed variables.

Model 1b is shown in Figure 3. The direct path from mutation to ASD was not significant; however, there was mediation through tuber count and seizure severity. TSC2 (vs. TSC1) mutation predicted higher tuber count and spasms severity. In turn, tuber count predicted spasms and other seizure types. Other seizures in the first two years of life were strongly associated with seizure severity at 7+ years of age, indicating considerable stability of seizure risk. Spasms (but not other seizure types) were associated with ASD severity, whereas both spasms and other seizure types were associated with IQ. This model accounts for correlation between spasms and other seizures in the first two years of life, and between ASD severity, IQ, and seizure severity at 7+ years of age. Panel B of Figure 3 shows two significant indirect pathways that mediate between mutation and ASD. In the first, mutation predicts tuber count, which in turn predicts spasms severity, and then ASD. In the second, mutation predicts spasms severity, which again predicts ASD. The pathways do not differ in magnitude (see Figure 3 legend), indicating that they contribute approximately equally to ASD risk. They converge on spasms, with the direct path from mutation to spasms accounting for 59% of the correlation between mutation and spasms, and the indirect path via tuber count accounting for 41%.

Model 2, which added age of seizure onset as a separate variable (Figure S18), provided a slightly worse fit to the data on some fits statistics and slightly better on others (Table 2). While earlier age of seizure onset was predicted by both mutation (β=-.30, *B*=-13.99, 95% *CI* -28.94–-2.35) and tuber count (β=-.29, *B*=-5.55, 95% *CI* -11.28–-1.81), it was not associated with ASD (β=.02, *B*=0.02, 95% *CI* -0.37–0.39), suggesting that age of onset does not mediate between mutation and ASD once severity of seizures in infancy are accounted for. Models 3a and 3b, which collapsed spasms and other seizure types together, did not fit the data well (significant chi-square statistic and RMSEA>0.08; Table 2).

DISCUSSION

This study aimed to disentangle the effects of risk factors for ASD operating during development in a unique cohort of children and young people with TSC. This was possible because of the strengths of the study, including: (i) a UK nationally representative sample of individuals with TSC; (ii) a longitudinal prospective design; (iii) ascertainment early in life; (iv) in-depth ASD assessments after the age of 7 years, including developmental history and observational assessments; (v) multiple methods, including cognitive and behavioural assessment, parent-report, clinical history, and neuroimaging; (vi) dimensional measures of risk factors and outcomes; (vii) differentiation of seizure types; and (viii) structural equation modelling (SEM) to explore the interplay of multiple risk factors over time.

The proportion of children meeting criteria for ASD (39.5%) was consistent with previous reports (Davis et al., 2015). Furthermore, a substantial proportion did not meet criteria but showed evidence of subclinical autistic-like difficulties, supporting the use of a dimensional measure. The SEM showed that the effect of mutation on ASD severity was mediated through pathways including tuber count and spasm severity. This could indicate severe spasms play a causal effect in neurodevelopment. Animal models show that recurrent or prolonged seizures alter expression of glutamate and GABA receptors, with potential long-term effects including lowered seizure threshold and cognitive impairment (Holmes, 2005). Longer duration (>3 weeks) of hypsarrhythmia (the EEG pattern associated with spasms) increases the risk of intellectual impairment (Primec, Stare, & Neubauer, 2006), as does longer duration to cessation of spasms and poor control of other seizure types after spasms (Goh, Kwiatkowski, Dorer, & Thiele, 2005). Severe spasms may act as a marker for a more severe and intractable epilepsy presentation that increases the risk for ASD (van Eeghen et al., 2013).

Seizures, including spasms, in the first 12 months of life increase risk of intellectual impairment and predict ASD risk behaviours at 12 and 24 months (Capal et al., 2017). This could indicate a sensitive period in brain development during the first year, when spasms are most likely to occur: there is a period of rapid functional maturation of the superior temporal and prefrontal cortices between 3 and 12 months of age (Lemaitre et al., 2021). Alternatively, spasms could be a marker for abnormalities that predispose to ASD, rather than playing a causal role. Children who later develop spasms have increased EEG connectivity in sleep, which may be a stable characteristic rather than EEG activity preceding spasms (Davis et al., 2019). Although based on small numbers, the existence of children in the current study with ASD but presumed seizure onset after the first two years of life indicates that seizures in infancy are not necessary for the development of ASD in TSC (Moavero et al., 2020; however, epileptiform electrophysiological disturbance may occur in the absence of overt seizures and EEG data are necessary to explore these issues). Epilepsy may exacerbate risk for adverse neurodevelopmental outcomes, rather than being the primary cause (Curatolo et al., 2016; Moavero et al., 2020). Preliminary data on whether early or preventative treatment of seizures with vigabatrin improves outcomes have been mixed, though there is evidence that rapid diagnosis and treatment of spasms improves development outcomes at 18 months (Davis et al., 2015; Moavero et al., 2020; O'Callaghan et al., 2018).

There was considerable stability of seizures over time and seizure severity at age 7+ years was correlated with ASD severity. Recent seizures or the effects of medication may have affected participants during assessment. If a participant had had a very recent seizure (e.g., earlier in the day), assessment was rescheduled. However, in individuals with poorly controlled epilepsy the effects of regular seizures and medication may be difficult to disentangle.

Seizures in TSC are thought to arise from tubers and perituberal cortex, although micropathological abnormalities extending beyond the tuber may play a role in epileptogenicity (Curatolo et al., 2016; Curatolo et al., 2015). If tubers are the main origin of seizures then the association between mutation and seizures should be mediated by tubers. The association between mutation and non-spasm seizures was entirely mediated by tuber count in the SEM. However, there was a significant direct path from mutation to spasm severity, accounting for 59% of the correlation between mutation and spasms. This suggests that the aetiology of spasms is partially independent of tuber count and may reflect disrupted connectivity secondary to more diffuse structural and functional abnormalities (Davis et al., 2015; Muhlebner, Bongaarts, Sarnat, Scholl, & Aronica, 2019).

ASD symptoms were inversely correlated with IQ. This has been reported elsewhere (van Eeghen et al., 2013), as has decline in nonverbal IQ from 12-36 months in infants with TSC who subsequently meet ASD criteria (Spurling Jeste et al., 2014). Shared genetic risks may make it difficult to separate ASD and intellectual disability (ID) in genetic disorders (Betancur, 2011; Thurm, Farmer, Salzman, Lord, & Bishop, 2019) and there may be shared risk pathways, such as severity of spasms being associated with both ASD and IQ in our models. Furthermore, ASD symptoms may have a detrimental effect on the attainment of cognitive and adaptive skills (Thurm et al., 2019) and lower IQ may reduce the capacity to compensate for ASD symptoms (Livingston, Colvert, Social Relationships Study, Bolton, & Happe, 2019). However, in the current study approximately a quarter of those meeting criteria for ASD had an estimated IQ >70 (Supplementary materials, Figure S17), showing that the phenotypes do not completely overlap (see also Curatolo, Napolioni, & Moavero, 2010). In this study, we modelled ASD and IQ as correlated outcomes, with the possibility of both overlapping and distinct predictors.

There are several limitations that should be considered. The SEM explained a relatively small proportion of variance of the ASD factor score, with 71% of variance explained by factors not included in the model. In clinical settings, prognosis is uncertain even with known risk factors (Mettin et al., 2014), showing the need to explore a wider range of risk factors. Tuber size (Koh et al., 2000) and 'tuber burden' (proportion of tuber volume to brain volume; Jansen et al., 2008) may be important, as are other grey and white matter lesions (Chou et al., 2008), and the location of lesions and epileptic foci (e.g., temporal lobe epileptic foci predicting ASD (Bolton et al., 2002); we did not find an association between presence of temporal lobe tubers and ASD, but did not have sufficient EEG data to study epileptic foci). Finally, environmental exposures and genetic background may also account for variability in outcomes, given the multifactorial nature of ASD.

Despite an attempt to recruit all cases of TSC newly diagnosed across the UK over a five year period, the resulting sample size is relatively small for SEM approaches. There was insufficient power to specify more complex models and paths with smaller coefficients were less likely to reach statistical significance. We retained these paths in the final model; some may reach significance with a larger sample.

Standardised ASD assessments like the ADI-R and ADOS-2 are not validated in children with physical disabilities, including visual and motor impairment, and caution is recommended when using diagnostic tools with children with very low IQ (Thurm et al., 2019). The ADI-R is designed to be used with children with a mental age of at least two years; however, in children with severe to profound ID, mental age may be lower than this at the age of 4-5 years when the diagnostic algorithm is focused. Given this, prevalence estimates for ASD should be interpreted cautiously. To address this in our models, we incorporated the ADI-R current behaviour algorithm and ADOS-2 scores in the ASD factor

score. This captured behaviour at a mean of 13 years (minimum 7.8 years); all except one child had a mental age of >2 years at assessment.

Caution may be warranted in using single gene disorders like TSC as a model for ASD, although the behavioural phenotype in TSC does not appear to differ from non-syndromic ASD (Jeste et al., 2016). The extent to which different underlying pathology converges on common pathways like disrupted connectivity – whether due to tubers, seizures, or microstructural abnormalities – is not yet clear. However, TSC has the potential to lead to insights and testable hypotheses about mechanisms leading to the ASD phenotype. There is substantial overlap in genes implicated in ASD, ID, and epilepsy (Betancur, 2011), suggesting that disorders like TSC that carry high risk of all three may have implications for ASD more broadly.

In summary, in this nationally representative cohort of children and young people with TSC, we demonstrated risk pathways from mutation to ASD symptoms, mediated by cortical tuber count and epileptic spasms in infancy. Seizures in infancy may increase ASD symptom severity and push genetically vulnerable individuals over the threshold for an ASD diagnosis (Hagerman, 2013). Recent treatment trials have been mixed, with evidence that early treatment of spasms may improve development outcomes (O'Callaghan et al., 2018) but also that early control of seizures does not significantly reduce ASD risk (Moavero et al., 2020); it is possible that spasms both act as a marker of underlying pathology and exacerbate risk. As ASD is increasingly recognised as the final common pathway for many genetic brain disorders and with substantial overlap in risks for ID and epilepsy (Betancur, 2011; Peng et al., 2021), exploring the possibility of common pathophysiological mechanisms underlying ASD, ID, and epilepsy may lead to important insights of wider applicability.

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Figure legends

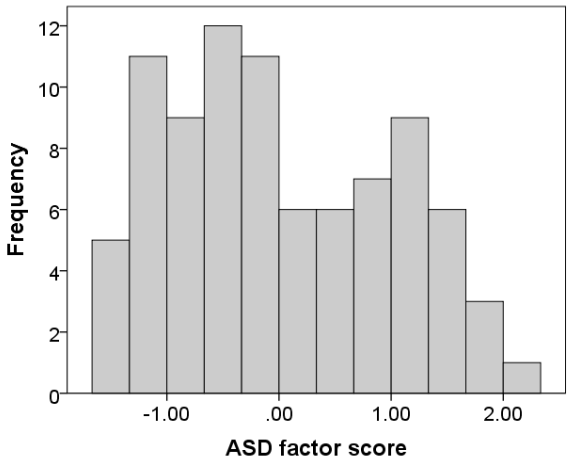

Figure 1. ASD latent factor score. Factor scores were based on ADI-R diagnostic algorithm, ADI-R current behaviour algorithm, and ADOS-2 Comparison Scores; *n*=86.

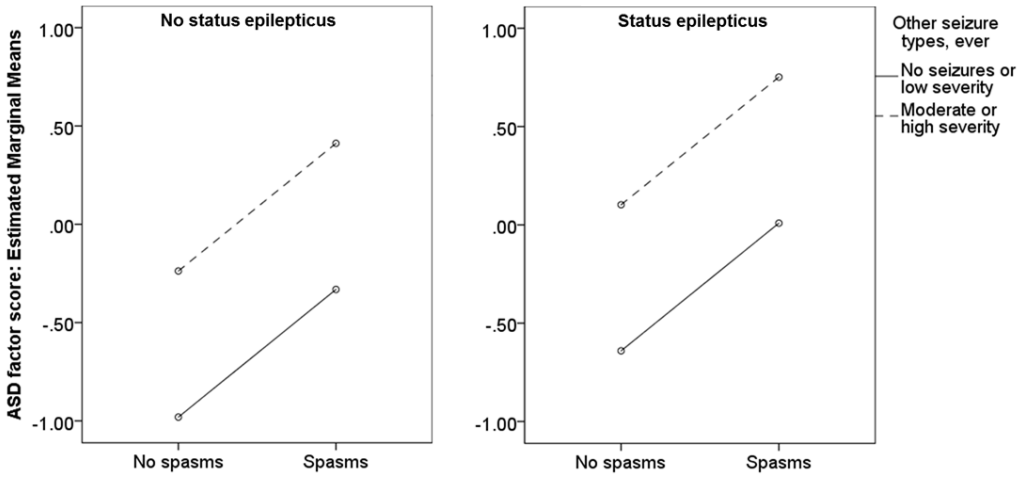

Figure 2. Estimated marginal means for ASD factor scores by epilepsy risk factors. Estimated marginal means from ANOVA with epileptic spasms (no spasms vs. history of spasms; F(df)=13.44(1,81), p<.001), status epilepticus (no status epilepticus vs. history of status epilepticus; F(df)=3.42(1,81), p=.068), and other seizure types severity (no seizures or low severity vs. moderate to high severity; F(df)=15.28(1,81), p<.001) as between subjects factors.

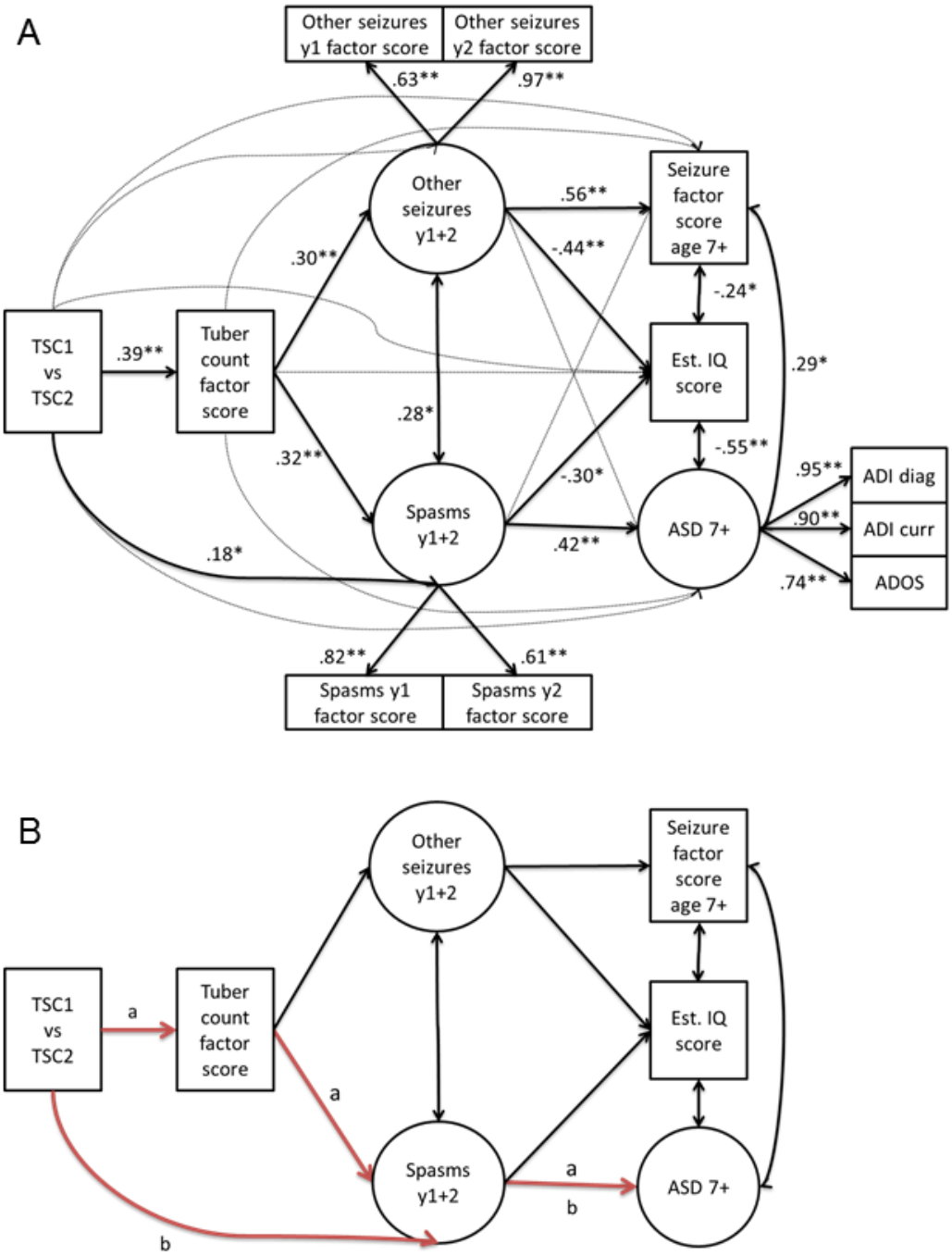

Figure 3. Best-fitting Structural Equation Model (SEM; model 1b) showing pathways mediating between mutation and ASD factor score. (**A**) SEM showing all paths included in the model; standardised path coefficients are only shown where the estimate was significant

(based on bootstrapped 95% confidence intervals); (**B**) Model showing significant indirect pathways between mutation and ASD factor score: *a* path (*mutation→tubers→spasms→ASD*), *B*=2.08 (95% *CI* 0.15–8.02); *b* path (*mutation→spasms→ASD*), *B*=2.98 (95% *CI* 0.04–8.89); overlapping confidence intervals indicate that the magnitude of these pathways does not differ. ADI diag=Autism Diagnostic Interview–Revised, Diagnostic Algorithm; ADI curr=Autism Diagnostic Interview–Revised, Current Behavior Algorithm; ADOS=Autism Diagnostic Observation Schedule (ADOS-2).

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