NEXMIF encephalopathy: an X-linked disorder with male and female phenotypic patterns

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Purpose: Pathogenic variants in the X-linked gene *NEXMIF* (previously *KIAA2022*) are associated with intellectual disability (ID), autism spectrum disorder, and epilepsy. We aimed to delineate the female and male phenotypic spectrum of *NEXMIF* encephalopathy.

Methods: Through an international collaboration, we analyzed the phenotypes and genotypes of 87 patients with *NEXMIF* encephalopathy.

Results: Sixty-three females and 24 males (46 new patients) with *NEXMIF* encephalopathy were studied, with 30 novel variants. Phenotypic features included developmental delay/ID in 86/87 (99%), seizures in 71/86 (83%) and multiple comorbidities. Generalized seizures predominated including myoclonic seizures and absence seizures (both 46/70, 66%), absence with eyelid myoclonia (17/70, 24%), and atonic seizures (30/70, 43%). Males had more severe developmental impairment; females had epilepsy more frequently, and varied from unaffected to severely affected.

All *NEXMIF* pathogenic variants led to a premature stop codon or were deleterious structural variants. Most arose de novo, although X-linked segregation occurred for both sexes. Somatic mosaicism occurred in two males and a family with suspected parental mosaicism.

Conclusion: *NEXMIF* encephalopathy is an X-linked, generalized developmental and epileptic encephalopathy characterized by myoclonic–atonic epilepsy overlapping with eyelid myoclonia with absence. Some patients have developmental encephalopathy without epilepsy. Males have more severe developmental impairment. *NEXMIF* encephalopathy arises due to loss-of-function variants.

INTRODUCTION

The developmental and epileptic encephalopathies (DEEs) are the most severe group of epilepsies in which frequent epileptic activity, in addition to the underlying etiology, contributes to developmental impairment, with onset typically in infancy or childhood.[1] At least 50% of the DEEs have a genetic cause,[2] and there is significant etiological overlap with other neurodevelopmental disorders such as intellectual disability (ID) and autism spectrum disorder (ASD).[3] A subgroup of neurodevelopmental disorders are X-linked where phenotypic expression may vary markedly between males and females. e.g.:[4,5] X-chromosome inactivation (XCI) is the epigenetic process where transcription of one of the X-chromosomes in female cells is silenced to balance gene dosage. This usually occurs in a random manner leading to cellular mosaicism. In females carrying a heterozygous X-chromosome pathogenic variant, skewing of XCI toward the wild type or mutant allele may protect them from, or predispose them to, disease.[6,7]

The Neurite EXtension and MIgration Factor (*NEXMIF*), previously called *KIAA2022* (OMIM 300524), is an X-linked gene that is thought to play an important role in early brain development.[8–11] Pathogenic *NEXMIF* variants were first identified in males with nonsyndromic X-linked ID with poor or absent speech, subtle dysmorphic features, and sometimes epilepsy.[12,13] Heterozygous females in these families were mostly unaffected. Subsequently, a few affected females have been described[14–16] and, in 2016, a series of 14 affected heterozygous females was reported.[17] Females typically had generalized epilepsy and less severe intellectual impairment than males. The authors suggested that phenotypic variation between females may be attributable to XCI.

Here, we aimed to systematically evaluate the phenotypic spectrum of *NEXMIF* encephalopathy focusing on the epileptology and to study genotype–phenotype correlations in males and females in a large international cohort of patients.

MATERIALS AND METHODS

Patient ascertainment and phenotyping
Patients with *NEXMIF* encephalopathy were recruited through the Epilepsy Genetics Research program at the University of Melbourne and a *NEXMIF* European collaboration. Epilepsy, developmental, and medical histories were collected, with review of medical files, electroencephalogram (EEG) and magnetic resonance imaging (MRI) studies, and administration of an epilepsy questionnaire where possible.

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Seizures were classified according to the 2017 International League Against Epilepsy classification[18] and, where possible, a specific epilepsy syndrome diagnosis was made.[1,19] Video EEG recordings and MRI images were reviewed by an electrophysiologist (EG) and pediatric neuroradiologist (SM), where available. Clinical photographs (17 new, 1 published without a picture,[20] 11 published cases[12,13,15,16,21,22]) were reviewed and analyzed through the program Face2Gene (see below). Unaffected heterozygous females were not included in the data.

Genotyping and X-inactivation

NEXMIF variants in new patients were identified by collaborating research and diagnostic laboratories. Parental and family segregation studies were performed where possible. Pathogenicity of variants was assessed according to the American College of Medical Genetics and Genomics guidelines.[23] Of 52 unreported patients, 46 carried pathogenic or likely pathogenic variants (43 singletons; 3 cases from one family) and 6 patients from 2 families carried variants of uncertain significance (VUS) reviewed separately. Due to the uncertain nature of both variants, we did not classify these individuals as having *NEXMIF* encephalopathy and did not include them in our analysis. All variants were absent in the Genome Aggregation Database (gnomAD).[24] *NEXMIF* transcript NM_001008537.3 was used for coding variant nomenclature (Supplementary Table S1). Where possible, XCI testing in affected females was performed in blood in the diagnostic and research laboratories that identified the variant. X-inactivation was interpreted as skewed when ratios were ≥75:25.[17]

Literature review

A PubMed search was performed with the search terms "*KIAA2022*" or "*NEXMIF*" (search ended 10 April 2020). We selected all publications in English reporting (possibly) pathogenic *NEXMIF* variants, and identified 14 studies, reporting 41 patients. For 38 patients, clinical and genetic data were available for systematic review.[12–17,21,22,25–30] For three reported patients, we collected additional clinical information.[26,29] *NEXMIF* pathogenic variants reported in large data set studies with minimal or no clinical information were not included.

Automated facial analysis

Frontal facial 2D photographs of 27 individuals with *NEXMIF* encephalopathy were analyzed using the Face2Gene Research application (FDNA Inc., Boston, MA).[31] This *NEXMIF* cohort was compared with 27 controls and 27 patients with *SYNGAP1* encephalopathy (Supplementary methods). Patients with *SYNGAP1* encephalopathy were chosen as their epileptology is similar to *NEXMIF* encephalopathy. Cohorts were matched for ethnicity, gender, and age.

Statistical analysis

All patients with pathogenic or likely pathogenic *NEXMIF* variants were included in our analyses. Only the index for each family was included in statistical analyses comparing males and females. Differences between males and females were calculated with a Fisher's exact test for binary variables or a Mann–Whitney U test for continuous and ordinal variables. Statistical analyses were performed using SPSS statistics software (IBM SPSS Statistics 26). All reported tests were performed two-tailed with an alpha level for significance of $p < 0.05$.

Ethics statement

Written informed consent for participation/publication was obtained from all participants or their parents or legal guardians in the case of minors or adults with ID. Separate consent forms for publication of pictures/videos were obtained when applicable. This study was approved by the Human Research Ethics Committees of Austin Health, Australia and Western Zealand, Denmark.

RESULTS

Our cohort comprised 87 patients (78 families) with *NEXMIF* encephalopathy including 46 new patients, and 3 published patients with additional information[26,29] (phenotypic data in Supplementary Table S2) and data from 38 published patients (Supplementary Table S3).[12–17,21,22,25,27,28,30] The cohort included 24 males and 63 females; median age at study was 10 years (range 1–53 years). As we did not have complete information on all patients, denominators indicate the number in whom information on the clinical feature addressed was available.

Neurodevelopment, autism spectrum disorder, and behavioral features

All 24 males had developmental delay, observed prior to 1 year of age in 15/18 (83%) (Table 1). Median age of walking was 34 months (range 14 months–6 years). Two boys could not walk at 3 years and one lost the ability to walk 6 months after starting walking.[13] Seven of 12 (58%) males older than 3 years were nonverbal, 3 (25%) spoke single words, and 2 (17%) spoke in simple sentences. Regression or stagnation in development occurred in 7/10 males (70%) at median age 22 months (range 5–40 months) and, in 5, coincided with seizure onset or increased seizure frequency. Seventeen of 23 (74%) males had severe to profound ID; 6/23 (26%) had mild to moderate ID.

Females with *NEXMIF* encephalopathy showed broader variation in developmental and cognitive profile. In comparison with males, onset of developmental delay was recognized later (<1 years in only 17/47, 36%, $p = 0.006$) with median age of walking significantly earlier at 18 months (range 11 months–4 years, $p < 0.001$). More females reached a higher level of expressive language, with 38/52 (73%) females older than 3 years speaking simple sentences ($p < 0.001$). Regression or stagnation in development occurred in 14/44 (32%) females, coinciding with seizure onset or increased seizure frequency in 5/9 females on whom this information was available. Accordingly, levels of ID ranged from mild to

Table 1 Developmental and epilepsy features of patients with *NEXMIF* encephalopathy comparing males and females.

	Males	Females	Statistical analyses (males vs. females)	Total cohort
Number of patients	$n = 24$	$n = 63$		$n = 87$
Age at last follow-up	Median: 10 years Range: 2–40 years ($n = 22$)	Median: 11 years Range: 1–53 years ($n = 63$)		Median: 10 years Range: 1–53 years ($n = 85$)
Development and behavior				
Age onset DD	$n = 18$	$n = 47$		$n = 65$
1 ≤ 1 year	15 (83%)	17 (36%)	$p = $ **0.006** (Mann–Whitney U)	32 (49%)
2 = 1–2 years	0 (0%)	19 (40%)		19 (29%)
3 = 2–3 years	2 (11%)	7 (15%)		9 (14%)
4 = 3–4 years	1 (6%)	3 (6%)		4 (6%)
5 = 4–5 years	0 (–)	0 (–)		0 (–)
6 = 5–6 years	0 (–)	1 (2%)		1 (2%)
Age at walking	Median: 34 months Range: 14–72 months ($n = 17$)	Median: 18 months Range: 11–48 months ($n = 54$)	$p \leq$ **0.001** (Mann–Whitney U)	Median: 18 months Range: 11–72 months ($n = 71$)
Expressive language score (age >3 years)	$n = 12$	$n = 52$		$n = 64$
1 = Nonverbal	7 (58%)	5 (10%)	$p \leq$ **0.001** (Mann–Whitney U)	12 (19%)
2 = Single words	3 (25%)	9 (17%)		12 (19%)
3 = (Simple) sentences	2 (17%)	38 (73%)		40 (63%)
Stagnation or regression	7 (70%, $n = 10$)	14 (32%, $n = 44$)		21 (39%, $n = 54$)
Cognitive score	$n = 23$	$n = 61$		$n = 84$
1 = Profound ID	7 (30%)	1 (2%)	$p \leq$ **0.001** (Mann–Whitney U)	8 (10%)
2 = Severe ID	10 (44%)	18 (30%)		28 (33%)
3 = Mild–moderate ID	6 (26%)	41 (67%)		47 (56%)
4 = No ID	0 (–)	1 (2%)		1 (1%)
ASD or autistic features	18 (75%, $n = 24$)	28 (46%, $n = 61$)	$p = 0.057$ (Fisher's exact)	46 (54%, $n = 85$)
Behavioral problems	19 (79%, $n = 24$)	10 (67%, $n = 61$)		60 (70%, $n = 85$)
Seizures	15 (65%, $n = 23$)	56 (89%, $n = 63$)	$p = $ **0.005** (Fisher's exact)	71 (83%, $n = 86$)
Age at onset	Median: 15 months Range: 1–168 months ($n = 15$)	Median: 19 months Range: 1–204 months ($n = 54$)	$p = 0.588$ (Mann–Whitney U)	Median: 19 months Range: 1–204 months ($n = 69$)
Seizure type	$n = 14$	$n = 56$		$n = 70$
Myoclonic	6 (40%)	40 (71%)		46 (66%)
(Myoclonic)–atonic	5 (33%)	25 (45%)		30 (43%)
Tonic–clonic	4 (27%)	24 (43%)		28 (40%)
Any absence	6 (40%)	40 (71%)		46 (66%)
Absences	6 (40%)	30 (54%)		36 (51%)
Atypical absence	2 (13%)	3 (5%)		5 (7%)
Absences with eyelid myoclonia	1 (7%)	16 (29%)		17 (24%)
Myoclonic absence	0 (–)	5 (9%)		5 (7%)
Tonic	2 (13%)	8 (14%)		10 (14%)
Clonic	0 (–)	3 (5%)		3 (4%)
Focal	2 (13%)	5 (9%)		7 (10%)
Epileptic spasms	5 (33%)	1 (2%)		6 (9%)
Febrile seizures	1 (7%)	1 (2%)		2 (3%)
Status epilepticus (SE, total)	$n = 15$	$n = 51$		$n = 66$
Nonconvulsive SE	2 (13%)	11 (22%)		13 (20%)
SE, not specified	0 (–)	4 (8%)		4 (6%)
Clinical seizure triggers	$n = 14$	$n = 56$		$n = 70$
Clinical photosensitivity	1 (7%)	6 (11%)		7 (10%)
Self-inducing	1 (7%)	3 (6%)		4 (6%)
Eye closure	0	5 (9%)		5 (7%)
EEG	$n = 14$	$n = 49$		$n = 63$
Generalized (poly)spike wave	5 (36%)	38 (78%)		43 (68%)
Paroxysmal fast activity	1 (7%)	8 (16%)		9 (14%)
Multifocal/focal discharges	1 (7%)	17 (35%)		18 (29%)
(Modified) hypsarrhythmia	3 (21%)	0 (0%)		3 (5%)
EEG triggers	$n = 14$	$n = 49$		$n = 63$
Intermittent photic stimulation	1 (7%)	9 (18%)		10 (16%)
Eye closure	1 (7%)	14 (29%)		15 (24%)
Fixation off sensitivity	0 (0%)	2 (4%)		2 (3%)
Hyperventilation	0 (0%)	7 (14%)		7 (11%)
Sleep	4 (29%)	18 (37%)		22 (35%)
Epilepsy syndrome (only novel cohort)	$n = 7$	$n = 35$		$n = 42$
Myoclonic–atonic epilepsy (MAE)	0 (0%)	7 (20%)		7 (17%)
Eyelid myoclonia with absence (EMA)	0 (0%)	10 (29%)		10 (24%)
MAE/EMA overlap	2 (29%)	3 (9%)		5 (12%)
Idiopathic generalized epilepsy	0 (0%)	1 (3%)		1 (2%)
West syndrome	1 (14%)	0 (0%)		1 (2%)
Epileptic spasms	1 (14%)	0 (0%)		1 (2%)
DEE, myoclonic	2 (29%)	10 (28%)		12 (29%)
Unclassified DEE	1 (14%)	4 (11%)		5 (12%)
Seizure-free	7 (47%, $n = 15$)	4 (7%, $n = 55$)	$p = 0.001$ (Fisher's exact)	11 (16%, $n = 70$)

Percentages relate to the number of patients for whom the information was available based on *n*). All patients carrying pathogenic/likely pathogenic *NEXMIF* variants are included in the overall numbers and percentages given in the columns "males," "females," and "total cohort" whereas only index patients were included for statistical comparisons between males and females. Significant *p* values are indicated in bold.

ASD autism spectrum disorder, *DD* developmental delay, *DEE* developmental and epileptic encephalopathy, *EEG* electroencephalogram, *ID* intellectual disability.

moderate in 41/61 (67%), to severe or profound ID in 19/61 (31%), the inverse pattern to the male cognitive profile ($p < 0.001$). Of note, there was 1/61 (2%) females who had generalized epilepsy without ID.[17] Only affected females were included in our analysis. Healthy heterozygous females were described in several published X-linked families with affected males only[12,13,25] and also found in our families (Supplementary Table S2).

In 22/54 (41%) patients with seizures on whom information was available, developmental delay preceded seizure onset. Moreover, 15/86 (17%) patients (8/23 [35%] males, 7/63 [11%] females) had developmental delay/ID without seizures.

Although ASD or autistic features were more frequently reported in males (18/24, 75%) than females (28/61, 46%), this difference did not reach statistical significance ($p = 0.057$) when including index patients only. In 60/85 (70%) patients, behavioral problems occurred, most frequently hyperactivity (25/85, 29%), often with attention deficit disorder (20/85, 24%), aggression (20/85, 24%), and temper tantrums (10/85, 12%). Stereotypies were observed in 13/85 (15%) patients.

Seizure semiology
Seizures were present in 71/86 (83%) patients, less frequently in males (15/23, 65%) than females (56/63, 89%, $p = 0.005$). One reported male had electrical seizures without clinical correlate and was not included in our analysis.[25] Seizure onset age did not significantly differ between males (median 15 months, range 1 month–14 years) and females (median 19 months, range 1 month–17 years, $p = 0.588$). Seizure types were predominantly generalized with different types of absence seizures in 46/70 (66%), including absence with eyelid myoclonia in 17/70 (24%), and myoclonic seizures in 46/70 (66%). Other frequently observed seizure types included myoclonic–atonic or atonic seizures in 30/70 (43%) and tonic–clonic seizures (28/70, 40%). Less frequently observed were tonic seizures (10/70, 14%), focal seizures (7/70, 10%), epileptic spasms (6/70, 9%), clonic seizures (3/70, 4%), and febrile seizures (2/70, 3%). Males showed a propensity to epileptic spasms (5/14 [36%] males versus 1/56 [2%] females). One reported male did not have seizures but had epileptiform activity on EEG.[30]

A striking feature was the occurrence of nonconvulsive status epilepticus (NCSE), with prominent myoclonic and absence components, in 13/66 (20%) patients. The most severely affected individuals had episodes of myoclonic status epilepticus lasting several hours that, in some individuals, occurred nightly (Supplementary video).

Environmental photic stimulation was described as a seizure trigger in 7/70 (10%) patients, with eye closure triggering seizures in 5/70 (7%) patients. Clinical photosensitivity was associated with self-induction of seizures (e.g., flickering of hands in front of the eyes) in 4/70 (6%) individuals. Other seizure triggers included stress, high environmental temperature or fever, and perimenstrual period.

Electroencephalography
EEG data (including 21 video EEG data) were available for 63/70 (90%) patients with epilepsy. The predominant findings were generalized spike wave or polyspike wave discharges in 43/63 (68%) patients (Fig. 1). This was accompanied by paroxysmal fast activity (PFA) in 9/63 (14%) patients. In 18/63 (29%) patients, multifocal, or, more rarely focal, epileptiform discharges were observed. In 22/63 (35%) patients, epileptiform activity markedly increased in sleep. Electroencephalographic photosensitivity was documented in 19/63 (30%) patients, consisting of eye closure sensitivity in 15/63 (24%) and/or sensitivity to intermittent photic stimulation (IPS) in 10/63 (16%). Fixation-off sensitivity occurred in 2/63 (3%). Three males had (modified) hypsarrhythmia.[13]

Epilepsy syndromes in new cohort
We were able to classify the epilepsy syndrome in 42/44 new patients based on the available data. The most frequent epilepsy syndromes were eyelid myoclonia with absence (EMA), described by Jeavons in 10 (24%, all female); myoclonic–atonic epilepsy (MAE), described by Doose in 7 (17%, all female); or patients with DEE overlapping these two syndromes in 5 (12%, 2 male, 3 female). We note that two girls classified as EMA had earlier onset (at 9 months and 1 year) than is usually accepted for this syndrome, though earlier onset has been observed in the setting of ID.[32] Further, one female had genetic generalized epilepsy with mild ID. Interestingly, West syndrome was diagnosed in one male and another male had epileptic spasms without hypsarrhythmia. Twelve of 42 (29%) patients had a nonsyndromic, myoclonic DEE, while 5/42 (12%) had an unclassified DEE.

Previously, an epilepsy syndrome has been described in only 5/26 published cases. This included MAE in 2 females,[17] EMA in one female,[20] West syndrome[13,28] and Lennox–Gastaut syndrome in one male each.[13] Two additional patients had epileptic spasms and hypsarrhythmia but were not classified as West syndrome.[13]

Seizure outcome
Of the 51 patients on whom information was available regarding treatment, 39/51 (76%) received three or more antiepileptic drugs (AEDs). Ten additional reported patients were drug-resistant, although the number of AEDs trialed was not documented. At last follow-up, 59/70 (84%) patients with seizures had ongoing seizures, while 11 were seizure-free (7/15; 47% males versus 4/55; 7% females, $p = 0.001$). Our retrospective analysis found that the AEDs reported most often to be effective, in various combinations, were valproate (21 patients), with or without lamotrigine (10 patients), levetiracetam (14 patients), and ethosuximide (15 patients), consistent with their use in generalized epilepsies.

Neurological examination
Neurological examination was normal in 45/85 (53%) patients. Abnormalities included hypotonia (32/85, 38%), poor coordination or ataxia (14/85, 16%), and hypertonia or
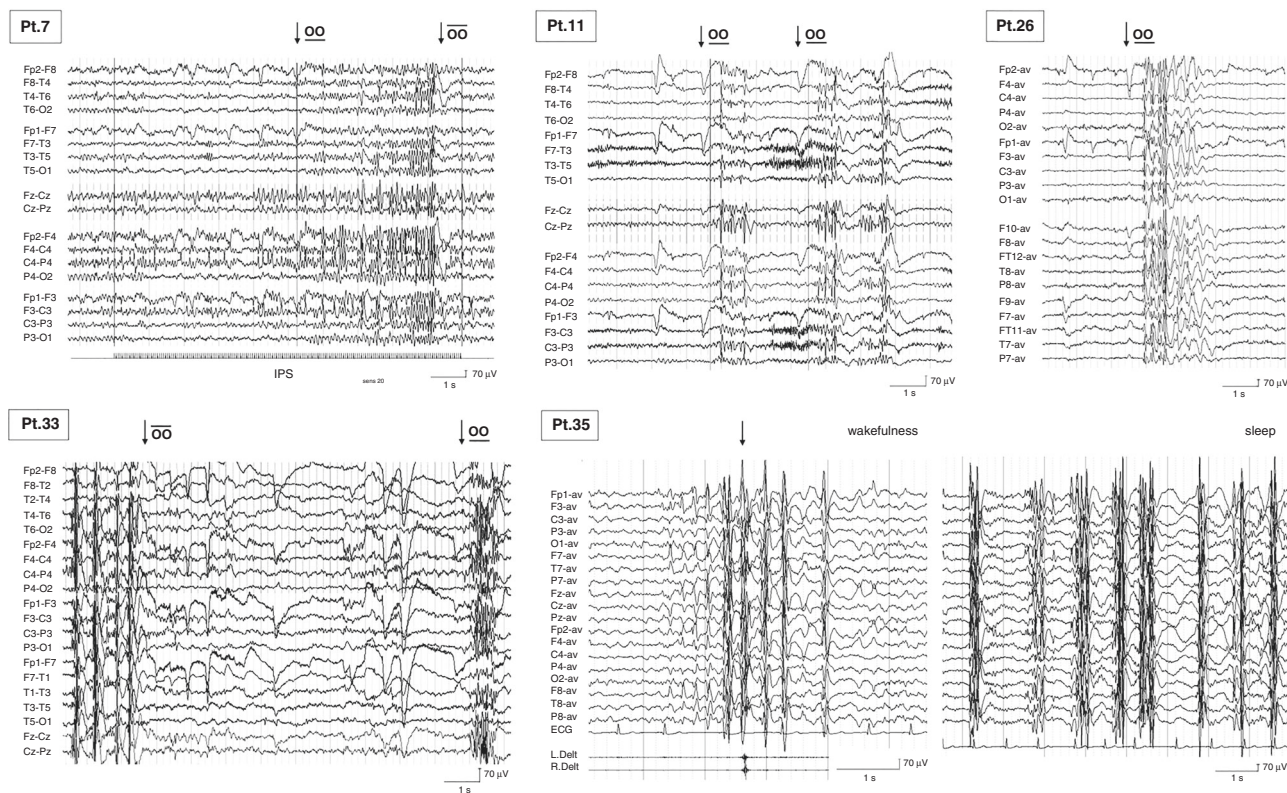
Fig. 1 Common electroencephalogram (EEG) features in *NEXMIF* encephalopathy. Interictal epileptiform abnormalities typically consist of generalized polyspike wave (patients 33 and 35) and diffuse rhythmic fast activity (patients 7 and 11) alone or in combination (patient 26). Polyspike wave complexes may be associated with a myoclonic seizure (arrow) and are often accentuated during non-REM sleep (patient 35). Eye closure sensitivity is a common feature (patients 7, 11, 26 and 33; denoted by OO). In one case (patient 11), subsequent trains of diffuse sharply contoured rhythmic activity (10–11 Hz) on eye closure were accompanied by almost continuous eyelid flutter and impaired awareness for more than 30 minutes, consistent with nonconvulsive status epilepticus.

spasticity (8/85, 9%). Microcephaly (≤2 SD below the mean) was more frequent in males (8/24; 33%) than females (6/60; 10%), although analysis of index patients alone did not show a significant difference ($p = 0.120$).

Dysmorphology including automated facial analysis
Information on dysmorphic features, including clinical pictures of 29 patients (11 published, Fig. 2), was available for 85/86 patients. Dysmorphic features were observed in 40/86 (47%) patients and were more frequent in males (16/23, 70%) than females (24/63, 38%, $p = 0.032$). When reviewing pictures of patients, most had subtle dysmorphic features, with hooded eyelids the most frequent characteristic (15/26; two cases from Kuroda et al.[21] omitted due to ethnicity).[21] Fewer than 10% of patients had other features such as abnormal ear placement, open hypotonic mouth, and narrow forehead.

Automated facial analysis of dysmorphic features could not distinguish our patient cohort from unaffected controls (whole cohort: area under the curve [AUC] of 0.617 [$p < 0.132$]; male cohort: AUC of 0.607 [$p < 0.245$]; female cohort: AUC of 0.654 [$p < 0.177$]) or from our previously reported *SYNGAP1* cohort (whole cohort: AUC of 0.643 [$p < 0.132$];

male cohort: AUC of 0.417 [$p < 0.546$]; female cohort: AUC of 0.647 [$p < 0.207$]).[33] (See Supplementary methods). Overall, no distinctive facial gestalt was identified clinically or via automated analysis.

Neuroimaging
Brain imaging was available for 78/87 patients. In 68/78 (87%), the MRI (performed at age 7 months to 25 years) was normal or showed minor findings. Ten of 78 (13%) patients had abnormalities, including regional or generalized brain atrophy in 5/78 (6%) (Supplementary Tables S2 and S3). Ten brain MRIs were available for analysis by a pediatric neuroradiologist (S. Mandelstam), and did not reveal any recurrent features (Supplementary Table S2).

Additional comorbidities
Additional comorbidities were identified in 62/86 (72%) patients. These most frequently affected the gastrointestinal system, with infantile gastroesophageal reflux occurring in 19/86 (22%) patients. Pre- or postnatal growth retardation (≤2 SD below the mean) was reported in 13/86 (15%) patients, more often affecting males (9/24, 38%) than females (4/62; 6%) with 11/13 having severe to profound ID. In contrast,

Fig. 2 *NEXMIF* dysmorphology. Pictures of new patients with *NEXMIF* encephalopathy, and the first photograph of a reported patient.[20] All patients are female, apart from male cases 1, 2, and 14. More frequent features include hooded eyelids, abnormal placement of the ears, open hypotonic mouth, thin upper lip, and narrow forehead.

about half of the adult patients were overweight or obese (11/18 [61%] females and 2/5 [40%] males). Strabismus was observed in 11/86 (13%; 9 male) patients. Dysfunction of the hypothalamic–pituitary axis occurred in 7/86 (8%; 4 females) patients: 1 had hypogonadotropic hypogonadism, 1 central hypothyroidism, 2 precocious puberty, 1 primary amenorrhea, and 2 delayed bone age.

Molecular genetics
Our cohort of 87 patients (78 families) had 58 unique *NEXMIF* variants including 28 stop gains, 24 frameshift variants, and 6 larger structural variants (Fig. 3). Thirty variants were novel. Ten variants were recurrent; five were new recurrent variants first identified in our cohort: p.(Trp112*) ($n = 2$), p.(Met295Valfs*2) ($n = 2$), p.(Tyr964*) ($n = 2$), p.(Ser995*) ($n = 2$), and p.(Asn1153Lysfs*8) ($n = 4$).

Fifty-nine of 78 (76%) families had de novo variants (51 females, 8 males) and 10 families had maternally inherited variants (new families with >2 affected individuals in Supplementary Fig. 1). Five of ten families included affected males only (and unaffected heterozygous females), and in five both females and males were affected. Half of the pathogenic small variants seen in families were also seen as de novo variants in sporadic cases. For three patients, only one parent was available and was shown to be negative for their child's variant. For three patients, parental DNA was not available.

Mosaicism was identified in blood-derived DNA in two male probands. Two sisters shared a recurrent variant and parental gonadal mosaicism was hypothesized, but was not detected in their parents' blood-derived DNA despite deep sequencing (coverage >200×).[29]

Genotype–phenotype correlation
Pathogenic *NEXMIF* variants included stopgain, frameshift, and larger structural variants; all were predicted to decrease protein expression. Smaller molecular variants were located in exon 3, the largest protein-encoding exon in *NEXMIF*. Missense pathogenic variants were not seen.

We did not expect genotype–phenotype correlations between patients with different variants, as all were predicted to lead to nonsense-mediated messenger RNA (mRNA) decay. The only possible exception was p.(Lys1396Argfs*3) as it is located in the last 10% of the coding region and may escape nonsense-mediated decay; however, the phenotype was consistent with our other cases. The main genotype–phenotype correlation in *NEXMIF* encephalopathy was related to sex, as detailed above. Interestingly, a reported male with a milder phenotype had an exon 2 duplication that led to a 60% reduction in *NEXMIF* expression, compared with the absence of expression shown in some males.[13]

XCI analysis was performed on 32/63 females (21 from new cohort, Supplementary Table 2). Seven females had a skewed XCI pattern. Comparing females with skewed versus females with random XCI, and also comparing sisters with skewed versus random XCI (families 4 and 7), there was no correlation between phenotype and XCI status. Two published females showed 100% skewed XCI and loss of *NEXMIF* expression and thus resembled males at the molecular level: one had a typical male phenotype (severe ID, ASD, no seizures), while the other's phenotype was consistent with either sex (moderate ID, no seizures, no autistic features). Interestingly, the two mosaic males (patients 2 and 47), whose genotype resembled an affected female, had moderate ID with worse seizure severity than most hemizygous males.
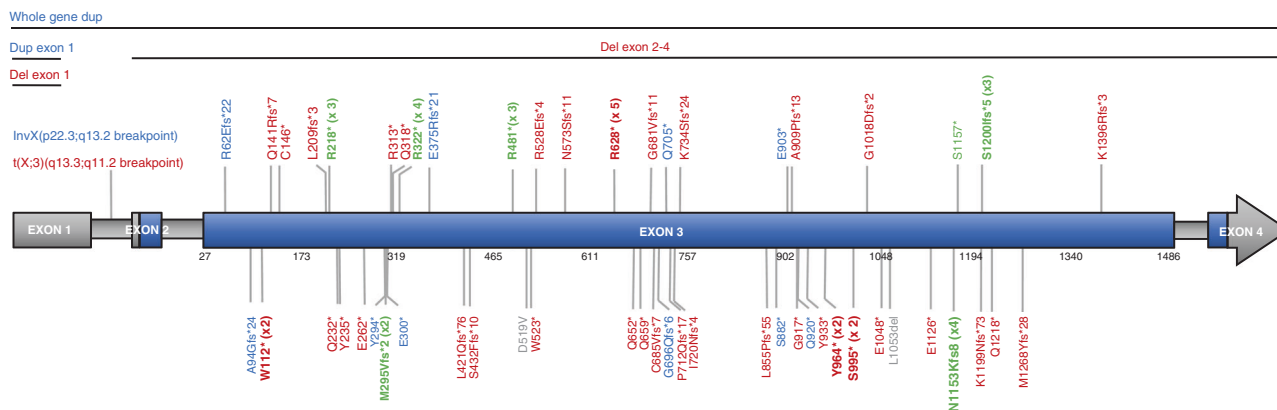
Fig. 3 *NEXMIF* variants with their genomic location. Genomic organization of *NEXMIF* with location of all pathogenic and likely pathogenic *NEXMIF* variants (based on Fig. **1** in de Lange et al.[17]). Coding regions are indicated in blue, untranslated regions in gray. All exons are shown at scale apart from exon 4, which continues as illustrated by the arrow. Previously reported variants are shown above the figure and newly identified variants below. Variants reported only in females are indicated in red, only in males in blue, and variants reported in both males and females in green. Recurrent variants are in bold; the number of independent families carrying the variant is shown in parentheses. Two variants of uncertain significance are indicated in grey.

Variants of uncertain significance

Two families with interesting VUS in *NEXMIF* were referred to our study (pedigrees in Supplementary Fig. 2; phenotypic data in Supplementary Table S2). One family had an inframe deletion p.(Leu1053del) and the other a missense variant p.(Asp519Val) predicted to be pathogenic by different in silico tools (Supplementary document); both variants were absent in gnomAD.[24] The inframe deletion was found in a family with three affected siblings and their unaffected mother, aunt, and grandmother. The variant was not found in their healthy brother. The missense variant was found in a family consisting of two half-brothers with ID, generalized epilepsy in one, and their mildly affected mother. Both families showed phenotypes in keeping with the *NEXMIF* encephalopathy phenotypic spectrum, suggesting that, with further functional evidence, they may be determined to be pathogenic.

DISCUSSION

In this study, we analyze the phenotypic and genetic features of a large cohort of 87 patients (78 families) with *NEXMIF* encephalopathy, including 46 new patients with 30 novel *NEXMIF* variants. All but 1[17] of 87 patients had cognitive impairment, with severe or profound ID in 43% of cases, with a male preponderance (74% males versus 31% females). ASD or autistic features and behavioral problems were common. Other neuropsychiatric features included stereotypies, and attention and hyperactivity problems.

Developmental delay occurred prior to seizure onset in 41% of patients with epilepsy, and 17% of patients did not have seizures. Regression or stagnation was reported in about a third, often coinciding with seizure onset or exacerbation.

Drug-resistant epilepsy was more frequent in females with *NEXMIF* encephalopathy. Nevertheless, one of our patients with the most severe seizure phenotype was male (supplementary video) and 7/8 males in our newly identified cohort had seizures, in contrast to "only" 8/15 published cases.

Ascertainment bias could have skewed the numbers in the original studies, which had fewer males with seizures, and conversely, in our cohort, as our patients were largely referred by epilepsy centers.

Seizure types were predominantly generalized, most frequently comprising myoclonic, absence of multiple types, atonic or myoclonic–atonic, and tonic–clonic seizures. One in five had a history of NCSE, often recurrent, with florid myoclonic components (Supplementary video). The EEG pattern showed irregular, bilaterally synchronous, symmetric spike wave and polyspike wave activity (Fig. **1**). Photosensitivity was observed as either a clinical or electroencephalographic phenomenon in about 30% of patients with epilepsy in response to variation in ambient light or intermittent photic stimulation, a feature typically associated with the genetic generalized epilepsies. Eye closure sensitivity on EEG, seen in 24% of *NEXMIF* epilepsy patients, is the hallmark of EMA.[34] It often occurs in individuals with photic sensitivity and involves a distinct pathophysiological network implicating both the occipital cortex and the supplementary motor area.[35]

NEXMIF encephalopathy presents a distinctive epilepsy syndrome of a myoclonic DEE comprising an overlap between the syndromes of MAE and EMA (Fig. **4**). A similar overlap of these specific epilepsy syndromes is seen in patients with pathogenic variants in *CHD2*,[36] *SYNGAP1*,[33] and *SLC6A1*.[37] This suggests that children with this phenotype should be tested for pathogenic variants in these genes. West syndrome or epileptic spasms, while rare, occurred predominantly in males. Several patients showed features reminiscent of Lennox–Gastaut syndrome, such as slow spike wave and generalized paroxysmal fast activity or tonic seizures.

Comorbidities were common in *NEXMIF* encephalopathy, including infantile hypotonia, ataxia, microcephaly, gastroesophageal reflux disease, strabismus, and dysmorphic features. Detailed clinical and digital analysis did not distinguish a characteristic facies. Growth retardation was more frequently observed in males, whereas obesity was noted
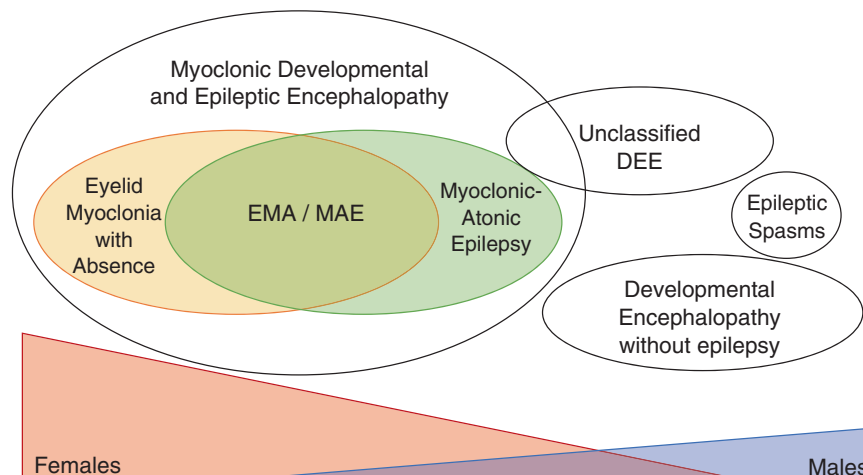
Fig. 4 NEXMIF encephalopathy: epilepsy syndromes. Conceptual figure showing epilepsy syndromes most frequently associated with *NEXMIF* encephalopathy and their frequency in females versus males. *DEE* developmental and epileptic encephalopathies, *EMA* eyelid myoclonia with absence, *MAE* myoclonic–atonic epilepsy.

in adult females. We observed endocrinological problems, including dysfunction of the hypothalamic–pituitary axis, in 8% of our cohort. As this is a small number, it may be serendipitous and unrelated to *NEXMIF* encephalopathy.

NEXMIF is an X-linked gene that is thought to play a role in neuronal morphogenesis, migration, and synapse formation, underscoring its critical function in normal brain development.[8–11] *NEXMIF* encephalopathy is caused by de novo or maternally inherited variants leading to a premature stop codon or larger structural variants. As such, the underlying mechanism is likely loss of function, ranging from total loss of function with no protein expression in hemizygous males, to partial loss of function with a variable decrease in protein expression in females due to XCI. This is supported by the constraint metrics in gnomAD that show that *NEXMIF* is loss-of-function intolerant (probability of loss of function intolerance [pLI] 1.00, o/e 0.06 [CI 0.03–0.2], http://gnomad.broadinstitute.org/ gene/ENSG00000050030:).[24] Further support can be drawn from the recent *Nexmif/Kidlia* knockout mouse model that recapitulated some of the human disease traits, such as learning deficits, decreased sociability, and seizures.[11] The role of inframe deletions and missense variants is unclear, albeit intriguing given the families described above, and requires interrogation with functional studies.

The most obvious genotype–phenotype correlation was that males were more severely affected than females. Males had more severe impairment with three-quarters having severe to profound ID. Epilepsy was more common in females, with females more likely to have EMA and MAE while males accounted for most cases of epileptic spasms. The majority of females had random XCI, not supporting the hypothesis that the XCI pattern in blood explains the phenotypic variation in females. Nevertheless, correlation of XCI status between blood and brain is uncertain, and we are not accessing the best tissue to explain neurological disease.

Other factors such as modifying genes and genetic background likely also influence phenotypic variability.

The hypothesis of cellular interference has been invoked in *PCHD19* X-linked clustering epilepsy.[4] *NEXMIF*, like *PCDH19*, is involved in cell–cell adhesion and neuronal migration.[8–10,17] Thus cellular mosaicism, occurring in females through XCI, could disrupt normal intercellular communication and neuronal networks, potentially predisposing females to epilepsy. This hypothesis is supported by our two males with somatic mosaic *NEXMIF* encephalopathy, who only had moderate ID and drug-resistant epilepsy, more closely resembling females with *NEXMIF* encephalopathy.

NEXMIF encephalopathy was initially described as an X-linked disorder in males.[12,13] With the identification of additional patients, 72% of cases are now female. A similar skewed sex distribution is observed in other dominant X-linked disorders, such as Rett syndrome associated with *MECP2* pathogenic variants. Different hypotheses could explain this skewed sex distribution for an X-linked disease:[38] (1) pathogenic variants increase the risk of prenatal lethality in males; (2) pathogenic variants occur predominantly in male germ cells, resulting in affected daughters, as occurs in Rett syndrome,[39] or (3) females have a statistically higher chance of a de novo X-linked variant because they have two X-chromosomes.

The majority of patients with *NEXMIF* encephalopathy had drug-resistant epilepsy. Future efforts to target precision therapies for *NEXMIF* encephalopathy must also focus on the developmental aspects and comorbidities of this severe disease, in addition to the seizure disorder. In Rett syndrome due to *MECP2* pathogenic variants, therapeutic strategies targeting XCI to upregulate gene expression are underway.[40] Similar strategies could be considered for females with *NEXMIF* pathogenic variants. In males, carrying only the mutant *NEXMIF* allele, different molecular approaches will need to be considered.

NEXMIF encephalopathy typically presents with one of two pictures: most commonly, a myoclonic developmental and epileptic encephalopathy associated with drug-resistant

generalized epilepsy and less frequently, a developmental encephalopathy without epilepsy, more frequent in males. The *NEXMIF* epilepsy syndrome is an overlap of MAE and EMA. This X-linked loss-of-function disorder presents with a broad phenotypic spectrum in females, in contrast to a more severe picture in males.

SUPPLEMENTARY INFORMATION
The online version of this article (https://doi.org/10.1038/s41436-020-00988-9) contains supplementary material, which is available to authorized users.

DISCLOSURE
R.G. has acted as an investigator for studies with Zogenix, Biocodex, Biomarin, UCB, Angelini, and Eisai Inc. He has been a speaker and on advisory boards for Zogenix, Biocodex, Novartis, Biomarin, GW Pharma, and Biocodex; serves/has served on the editorial boards of *Epilepsia*, *Progress in Epileptic Disorders*, *Neuropediatrics*, *Journal of Child Neurology*, *Seizure*, *BMC Medical Genetics*, *Topics in Epilepsy*, and *Neurology*. He receives/has received research support from the Italian Ministry of Health, The European Community, The Tuscany Region, the Mariani Foundation, The Pisa Foundation, The Fund of Epilepsy, The GKT Special Trustees, The Italian Federation for Epilepsy, and The Italian Association for Epilepsy. I.E.S. serves/has served on the editorial boards of the *Annals of Neurology*, *Neurology*, and *Epileptic Disorders*; may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; has a patent for *SCN1A* testing held by Bionomics, Inc., and licensed to various diagnostic companies; has a patent molecular diagnostic/theranostic target for benign familial infantile epilepsy (BFIE) [PRRT2] 2011904493 & 2012900190 and PCT/AU2012/001321 (TECH ID:2012-009) with royalties paid. She has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia, Rogcon, and Xenon Pharmaceuticals; has received speaker honoraria from GlaxoSmithKline, UCB, BioMarin, Biocodex, and Eisai; has received funding for travel from UCB, Biocodex, GlaxoSmithKline, Biomarin, and Eisai; has served as an investigator for Zogenix, Zynerba, Ultragenyx, GW Pharma, UCB, Eisai, Anavex Life Scienand Marinus; and has consulted for Zynerba Pharmaceuticals, Atheneum Partners, Ovid Therapeutics, and UCB. She receives/has received research support from the National Health and Medical Research Council of Australia, Health Research Council of New Zealand, CURE, Australian Epilepsy Research Fund, March of Dimes and NIH/NINDS. D.M.A. has consulted for Eisai, UCB, and is part of the MAB of Stoke Therapeutics and DSF. B.G. has received consultancy fees from GW Pharmaceuticals companies, Zogenix, and Ovid/Takeda; and is a principal investigator for GW Research Ltd, Zogenix, LivaNova, Marinus Pharmaceuticals, and UCB. L.G.S. reports grants from Health Research Council of New Zealand, grants from Cure Kids New Zealand, during the conduct of the study; grants and personal fees from Zynerba, other from Sequirus, other from Nutricia, outside the submitted work. S.M.Z. has received research funding from Epilepsy Research UK, UCB Pharma, Glasgow Children's Hospital Charity, Dravet Syndrome UK. He serves as Editor-in-Chief of *European Journal of Pediatric Neurology* for which he receives an honorarium from Elsevier Ltd. He has participated in educational symposia/advisory boards/consulted for GW Pharma, Zogenix Ltd, Biocodex, Nutricia, and Encoded Genomics. The other authors declare no conflicts of interest.

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