Biallelic *MFSD2A* variants associated with congenital microcephaly, developmental delay, and recognizable neuroimaging features: expanding the phenotypic spectrum.

Marcello Scala[1,2,3¶], Geok Lin Chua[4¶], Cheen Fei Chin[4], Hessa S Alsaif[5], Borovikov Artem[6], Saima Riazuddin[7], Sheikh Riazuddin[8,9], Chiara Manzini[10], Mariasavina Severino[11], Alvin Kuk[4], Hao Fan[12,13,14], Yalda Jamshidi[15], Mehran Beiraghi Toosi[15], Mohammad Doosti[15], Ehsan Ghayoor Karimiani[15], Emer O'Connor[1], Yasser Sabr[16], Abdullah Alfaifi[17], Farah Ashrafzadeh[18], Pasquale Striano[2,3], Federico Zara[19], Fowzan S Alkuraya[20,21], Henry Houlden[1], Reza Maroofian[5*], David L. Silver[4*].

[1] Department of Neuromuscular Disorders, Institute of Neurology, University College London, London, United Kingdom

[2] Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa, Genoa, Italy

[3] Pediatric Neurology and Muscular Diseases Unit, IRCCS Istituto Giannina Gaslini, Genoa, Italy

[4] Signature Research Program in Cardiovascular and Metabolic Disorders, Duke-NUS Medical School, Singapore, 169857, Singapore

[5] Department of Genetics, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

[6] Research Centre for Medical Genetics, Moscow, Russia

[7] Department of Otorhinolaryngology Head & Neck Surgery, School of Medicine, University of Maryland, Baltimore, MD 21201, USA

[8] Center for Genetic Diseases, Shaheed Zulfiqar Ali Bhutto Medical University, Pakistan Institute of Medical Sciences, Islamabad, Pakistan

[9] National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore 53700, Pakistan

[10] Genetics Research Centre, Molecular and Clinical Sciences Institute, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK

[11] Neuroradiology Unit, IRCCS Istituto Giannina Gaslini, Genoa, Italy

[12] Bioinformatics Institute, Agency for Science, Technology and Research (A*STAR), 30 Biopolis St., Matrix No. 07-01, Singapore, 138671, Singapore

[13] Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore, 117543, Singapore.

[14] Centre for Computational Biology, DUKE-NUS Medical School, 8 College Road, Singapore, 169857, Singapore

[15] Department of pediatric diseases, Faculty of medicine, Mashhad University of Medical Sciences, Mashhad Iran

[16] Department of Obstetrics and Gynecology, King Saudi University, Riyadh, Saudi Arabia

[17] Pediatrics Department, Security Forces Hospital, Riyadh, Saudi Arabia

[18] Department of Pediatric Diseases, Mashhad University of Medical Sciences, Mashhad, Iran

[19] Laboratory of Neurogenetics and Neuroscience, IRCCS Istituto Giannina Gaslini, Genova Italy

[20] Department of Genetics, King Faisal Specialist Hospital and Research Center, Saudi Arabia

[21] Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh, Saudi Arabia

¶ These authors contributed equally

* Correspondence should be addressed to R.M. (rmaroofian@gmail.com) and D.L.S. (david.silver@duke-nus.edu.sg).

Abstract

Purpose: Major Facilitator Superfamily Domain containing 2a (*MFSD2A*) is an essential endothelial lipid transporter at the blood-brain barrier (BBB). Biallelic pathogenic variants in *MFSD2A* are associated with neurodevelopmental abnormalities in human. We sought to expand our knowledge of the phenotypic spectrum of *MFSD2A*-related disorder and demonstrate the mechanism of inactivation of the MFSD2A transporter underlying these conditions.

Methods: We carried out detailed analysis of the clinical and neuroradiological features of eight individuals from seven consanguineous families with severe microcephaly and psychomotor delay. Genetic investigation was performed through exome sequencing (ES). Structural insights on the human Mfsd2a model together with in-vitro biochemical assays were used to investigate the functional impact of the identified variants.

Results: All patients had primary microcephaly and severe developmental delay (DD). Brain MRI showed variable degrees of white matter reduction, ventricular enlargement, callosal hypodysgenesis, and pontine and vermian hypoplasia. ES led to the identification of biallelic *MFSD2A* variants in all subjects. The novel variants p.(Pro493Leu), p.(Pro164Thr), p.(Arg326His), p.(Val250Phe), and two novel frameshift variants p.(Gln462HisfsTer17) and p.(Cys251SerFsTer3), and the recurrent variants p.(Thr198Met) and p.(Thr159Met) resulted in either reduced MFSD2A protein expression and/or transport activity.

Conclusion: Our cohort contributes to further delineate the phenotypic spectrum of *MFSD2A*-related disorders, refining the neuroradiological characterization of this condition and supporting the role of *MFSD2A* in human brain development.

Keywords: ***MFSD2A*; microcephaly; developmental delay; brain MRI.**

INTRODUCTION

Major Facilitator Superfamily Domain containing 2a (*MFSD2A*) is a sodium-dependent lysophosphatidylcholine (LPC) transporter that is highly expressed at the endothelium of the blood-brain barrier (BBB).[1] Omega-3 fatty acids and other mono- and polyunsaturated fatty acids conjugated as LPCs are transported by MFSD2A, which plays a pivotal role in the supply of omega-3 fatty acids to the brain[1]. The essential role of *MFSD2A* in regulating lipogenesis in the developing brain has been recently demonstrated using loss-of-function mouse models.[2]

Five distinct homozygous loss-of-function *MFSD2A* variants have been reported in patients with neurodevelopmental abnormalities from seven consanguineous families. These patients showed developmental delay (DD), microcephaly, and neuroimaging abnormalities such as ventriculomegaly and hypoplasia of the corpus callosum, brainstem, and cerebellum. These observations underscored the fundamental role of LPC transport at the BBB for human brain development and clarified the structure-function relationships in the MFSD2A-mediated transport mechanism.[3-8]

In this study, we present seven new families with biallelic *MFSD2A* pathogenic variants, expanding the phenotype and defining the characteristic neuroimaging features of *MFSD2A*-related neurodevelopmental disorder, also known as Autosomal Recessive Microcephaly 15, (MCPH15). We provide clinical, genetic, and functional characterization of these novel variants and the previously reported p.(Thr198Met) variant on the transporter activity, which further substantiates the functional importance of LPC transport for human brain development.

MATERIALS AND METHODS

Subject ascertainment

This study was conducted in accordance with the Declaration of Helsinki. After informed consent was obtained from parents, clinical and genetic data were collected from the enrolled families.

Brain MRI

Brain MRI were acquired in different centers with different protocols, but all included diffusion weighted images, T1 and T2-weighted, and FLAIR images on the 3 planes. A pediatric neuroradiologist with 10 years of experience (MsS) and a pediatrician with expertise in neurogenetics (MS) reviewed the images in consensus.

Exome Sequencing

After standard DNA extraction from peripheral blood, proband exome sequencing (ES) was performed in all the families as previously described.[9-11] Variants were filtered out according to frequency, conservation, and predicted impact on protein function by several bioinformatic tools (SIFT, Polyphen-2, Mutation Taster). Candidate variants were subsequently validated through co-segregation studies by Sanger sequencing. All the variants are reported according to the NM_032793.5 transcript. GeneMatcher was used for the distributed case-matching.[12] Further details available in the Supplementary Methods.

Functional tests summary methods

Site-directed mutagenesis was used to create the Mfsd2a variants (Pro493Leu (P493L), Thr198Met (T198M), Pro164Thr (P164T), Arg326His (R326H) and Val250Phe (V250F)) in a mammalian expression vector, which were used to determine the effects on transporter function in mammalian cells. The amino acid variants in Mfsd2a protein were modeled and visualized to understand the causative mechanism of transporter dysfunction. Further details are available in the Supplementary Methods.

RESULTS

Clinical features

We present eight patients (Supplementary Table 1) from seven consanguineous families of varying ancestry: Saudi, Iranian, Pakistani, and Russian (Fig. 1a).

Patient 1 (Family A) is a 4-year-old female born to consanguineous parents (first-cousins) of Iranian ancestry. Prenatal ultrasound revealed microcephaly. At birth, her occipital frontal circumference (OFC) was 28 cm (-4.6 SDS). At the age of 6 months, she had head-lag, was unable to roll over, and lacked babbling. At 1 year of age, she started to suffer from myoclonic seizures and failure to thrive (FTT) due to dysphagia. Physical examination at 4 years showed progressive microcephaly with an OFC of 41 cm (-5.6 SDS) and bilateral talipes equinovarus (TEV). She was unable to walk and neurological examination revealed spastic quadriparesis and hyperreflexia. Karyotyping and metabolic testing were normal.

Patient 2 (Family B) is 4-year-old Iranian male born to consanguineous parents. Family history revealed several previous miscarriages. His older brother was healthy. At birth, his OFC was 27 cm (-3.9 SDS). He was diagnosed with global DD during infancy and started to suffer

from generalized tonic-clonic seizures since the age of 2 years. At 4 years, he was unable to sit and his language was very limited. Physical examination revealed bilateral TEV, progressive microcephaly with OFC of 37 cm (-8.8 SDS) and spastic quadriparesis (Fig. 1b).

Patient 3 and 4 (Family C) belong to a consanguineous family of Pakistani descent consisting of six siblings. Two males died in the neonatal period and were reported to have microcephaly. The proband (patient 3), a 17-year-old female, and her sister (patient 4), currently 27 years old, presented with severe global DD and aggressive behavior during infancy. They had no seizure history. Physical evaluation revealed mild muscle weakness, language limited to few words, and severe microcephaly, with an OFC of 49 cm (-5.0 SDS) and 47 cm (-6.9 SDS) in patients 3 and 4, respectively.

Patient 5 (Family D) is the youngest of two siblings born to unrelated parents of Russian descent. Neonatal history was unremarkable except for microcephaly. The baby started to suffer from generalized tonic-clonic seizures at the age of 4 months. Global DD was subsequently diagnosed at 1 year of age as he was unable to sit without support and had severe speech delay. At 5 years, the patient was unable to walk and could only say few words. He had microcephaly with OFC of 46 cm (-3.6 SDS), gross and fine motor impairment, and axial hypotonia. He also had dysphagia, excessive drooling, and some dysmorphic features, including wide nasal bridge and prominent epicanthal folds.

Patient 6 (Family E) is a 1-month-old Saudi female born to consanguineous parents. She was the youngest of four siblings. Her older brother had microcephaly but died during infancy. The patient was diagnosed with severe microcephaly at birth, with an OFC of 28.5 cm (-6.2 SDS). During the neonatal period she suffered from FTT due to severe dysphagia and physical examination further revealed generalized spasticity.

Patient 7 (Family F) is a 2-year-old male born to consanguineous parents from Saudi Arabia. During the neonatal period, he suffered from FTT and received percutaneous endoscopic gastrostomy (PEG) due to severe dysphagia. At 1 year of age, he started to suffer from recurrent seizures treated with phenobarbital and sodium valproate. Developmental milestones were severely delayed. The patient was also diagnosed with gastro-esophageal reflux. Physical examination showed microcephaly, bilateral TEV, generalized muscle weakness, and spasticity.

Patient 8 (Family G) is a 4-month-old female born to consanguineous Saudi parents. Prenatal ultrasound showed microcephaly and foetal echogenic bowel. Perinatal course was uneventful, but at the age of 1 week the baby was admitted to neonatal intensive care unit due to relevant feeding difficulties. At 4 months, she started to suffer from seizures requiring hospitalization. Physical examination showed microcephaly, generalized spasticity, bilateral hip dislocation, and left TEV.

Neuroimaging

Brain MRI revealed mild to severe white matter reduction with consequent ventricular dilatation in all subjects. In particular, patients the supratentorial white matter was markedly thinned in 5/8 patients, with severe ventriculomegaly. The degree of myelination was appropriate for the age in all subjects. The cortical gyral pattern was mildly to severely simplified in all cases, without other associated cortical malformations. The thalami were small and the corpus callosum was abnormal in all patients. In particular, in 5 subjects the corpus callosum was markedly thin and short, in 2 patients there was hypoplasia of the anterior portion of the corpus callosum, while in the remaining patient it was globally thin. Of note, the cingulate gyrus was present in all subjects.

Finally, inferior vermian hypoplasia was observed in all cases, while pontine hypoplasia was present in 6/8 patients.

Genetic findings

After filtering for allele frequency, conservation, and predicted functional impact, biallelic *MFSD2A* variants were prioritized as candidate disease-causing variants. Eight different variants were identified, including three homozygous missense variants (patient 1: c.1478 C>T, p.[Pro493Leu]; patient 3 and 4: c.593 C>T, p.[Thr198Met]; patient 6: c.476 C>T, p.[Thr159Met]), a homozygous splice site variant (patient 2: c.556+1 G>A), two homozygous frameshift variants (patient 7: c.1386_1435 del, p.[Gln462HisfsTer17]; patient 8: c.750_753 delCTGT, p.[Cys251SerfsTer3]), and two compound heterozygous missense variants (patient 5: c.748 G>T, p.[Val250Phe] and c.977 G>A, p.[Arg326His]) (Supplementary Table 2). Biparental segregation confirmed the autosomal recessive inheritance model. All variants are absent in the homozygous state and extremely rare in the heterozygous state in the most common population databases (including our database of 10,000 exomes, gnomAD, Greater Middle East Variome - GME, Iranome, and Ensembl). Missense variants were located at the amino acid residues with high levels of conservation, with a Genomic Evolutionary Rate Profiling (GERP) score between 5.49 to 5.94. The predicted effect on protein function was also consistent with a loss-of-function mechanism, with a CADD score of the variants ranging from 24.4 to 34. The two frameshift variants are predicted to result in nonsense mediated mRNA decay, likely leading to a functional knock-out. All the identified variants are predicted to be damaging by several bioinformatic tools, such as SIFT, Polyphen-2, and Mutation Taster. The splicing variant c.556+1 G>A is predicted

to result in aberrant splicing through the alteration of the wildtype (WT) donor site by Human Splice Finder and Variant Effect Predictor.

Mfsd2a variants lead to loss-of-function and/or loss-of-expression

Human Mfsd2a is a 530 amino acid glycosylated sodium-dependent MFS transporter composed of 12 conserved transmembrane domains.[7] To understand the consequence of P493L, P164T, T198M, R326H, and V250F variants on the structure and function of Mfsd2a, we utilized a published structural model of human Mfsd2a to carry out bioinformatic predictions.[7] In the T198M mutant model, M198 faces the internal cavity of the transporter and forms more favorable hydrophobic interactions with neighboring residues such as F399 from helix X, in comparison to T198 in the WT model that faces the membrane exterior (Fig. 1e). In the P493L mutant model, the proline-to-leucine amino acid change results in the extension of helix XII that is stabilized by a hydrophobic cluster formed by sidechains of L493 and three other residues Y294, L297, and F489 (Fig. 1e). In addition, multiple polar interactions observed in the WT model are absent in the P493L mutant model, including the hydrogen bonding interaction between Y294 and E497 as well as ionic locks between R498 and a negatively charged surface comprising D408, D411, and D412. These ionic locks were previously suggested to be important for the transporter function.[7] Taken together, we observed enhanced hydrophobic packing in both mutant models likely leading to increased structure rigidity and reduced mobility of the transporter, indirectly inactivating the transport of substrate. Additionally, the P493L mutant would be predicted to show a reduction in transport due to the partial loss of ionic locks.

We next utilized HEK293 cells, which do not endogenously express Mfsd2a, as an in vitro cell system to determine if Mfsd2a variants affect protein expression, localization, and transport function. Mock transfected and the sodium binding transporter inactive mutant Asp97Ala (D97A) served as negative controls,[1,7] while WT Mfsd2a served as a positive control. Western blot analysis of WT Mfsd2a showed the multiple protein bands similar to results previously reported for overexpression of Mfsd2a in HEK293 cells,[3,4,6] while all five mutants (P493L, T198M, P164T, R326H, and V250F) were expressed at less than 30% of WT Mfsd2a. This low level of protein expression of these five Mfsd2a mutants is consistent with predicted negative effects of these variants on protein folding (Fig. 1e). Despite low level expression of all five Mfsd2a mutants, immunofluorescence microscopy indicated that all mutants were expressed at the plasma membrane similarly to WT (Fig. 2b).

To directly test the functional consequences of these five variants on LPC transport, we utilized an established transport assay that quantifies net transport of ^{14}C-LPC-DHA in HEK293 cells. In order to directly compare transport activity between WT and T198M, P164L, P493L, R326H, and V250F mutants, it is important to first titrate down the amount of plasmid DNA for WT Mfsd2a transfected into cells in order to obtain a comparable expression level of WT to all five mutants. We found that 0.1 μg of WT yielded similarly low levels of expression of cells transfected with 2 μg of mutants (Fig. 2c). Surprisingly, at comparable protein expression levels of WT and mutants, four of the five mutants demonstrated comparable transport of 14C-LPC-DHA in HEK293 cells with T198M at 75%, P164T at 82%, R326H at 104%, and V250F at 80% of WT transport activity. Only P493L was similar to non-functional D97A negative control, indicating it is inactive (Fig. 2d).

Previously reported non-synonymous variants in Mfsd2a,3,4,6 have been shown to affect

transport function but not protein expression. In our cases, five of the novel pathogenic variants (T198M, P164T, R326H, V250F, and P493L) were extremely lowly expressed. Our findings support the conclusion that poor expression of Mfsd2a, despite normal transporter activity, can also be an underlying cause for severe microcephaly and hypomyelination in these patients, which further defines the etiology of Mfsd2a-related microcephaly.

DISCUSSION

MFSD2A is a sodium-dependent 12-pass transmembrane protein belonging to the major facilitator superfamily of secondary transporters. Mfsd2a plays a pivotal role at the BBB for the transport of plasma-derived LPCs conjugated to polyunsaturated fatty acids such as the omega-3 fatty acid docosahexaenoic acid (DHA) to the brain.[1,2,13] The deficiency of the DHA in the brain of *Mfsd2a*-knockout mice is associated with a severe neurodevelopmental phenotype characterized by microcephaly, cognitive impairment, ataxia, and severe anxiety.[11] In particular, microcephaly is likely explained by the fact that LPC transport not only provides accretion of DHA by the developing brain, but is also critical for providing LPC as building blocks for neuron arborization and regulation of membrane phospholipid composition.[2,5,14] The reports of loss-of-function *MFSD2A* variants in patients with a progressive microcephaly syndrome with severe ID and neuroimaging abnormalities has further supported the relevant role of this lipid transporter in human brain development and functioning.[3,4] The relevance of proper DHA metabolism for brain development and functioning is further supported by CYP2U1 deficiency. This enzyme is a member of the cytochrome P450 family 2 subfamily U and catalyzes the hydroxylation of arachidonic acid (AA) and AA-related long-chain fatty acids, including DHA.[15]

Biallelic loss-of-function *CYP2U1* variants cause spastic paraplegia 56 (SPG56), a complex neurological condition characterized by spasticity, cognitive impairment, and white matter abnormalities.[15]

Here, we present seven families with eight distinct loss-of-function variants in *MFSD2A*, including seven novel pathogenic variants. Patients 6 and 7 were briefly reported before by Shaheen et al. and Monies et al., respectively.[16,17] In line with previously reported cases, our patients showed a complex neurodevelopmental phenotype primarily characterized by severe progressive microcephaly, ID, spasticity, and speech delay (Supplementary Table 1) (Fig. 1f).[3,6,8] Less common clinical features were also identified in our cohort, including axial hypotonia, increased deep tendon reflexes, and seizures (Fig. 1b).[3,4,6-8] Of note, none of our patients died prematurely, although some of their siblings who died prematurely were most likely affected by the same condition. The longest follow-up was 27 years (patient 4), allowing assessment of the progression of microcephaly over time. Language was delayed in most subjects and one patient was nonverbal. Four patients showed skeletal abnormalities consistent with TEV. Dysmorphic features were observed in patient 5 only.

In previously reported cases, brain MRI revealed a spectrum of abnormal findings, including ventricular enlargement secondary to white matter paucity and hypoplasia of the corpus callosum, cerebellum, and brainstem.[3,4] In our study, we provide further evidence that affected subjects present severe microcephaly with simplified gyral pattern, associated with variable degrees of white matter reduction leading to mild to severe ventricular dilatation. Of note, the myelination was always appropriate for patients' age in our series, ruling out a hypomyelinating disorder. Interestingly, the corpus callosum was always abnormal, with severe hypodysplasia in most subjects. However, the cingulate gyrus was present in the most severe

cases as well, indicating that the corpus callosum was initially formed. Finally, the inferior cerebellar vermis was small in all subjects while hypoplasia of the pons was noted in almost all of them. Taken together, these neuroimaging features are consistent with an early prenatal developmental disruption and likely suggest a relevant role of LPCs in the development of both the cerebral gray and white matter.

In conclusion, our observations expand the phenotypic spectrum of *MFSD2A*-related microcephaly syndrome, providing new insights into the pathogenic mechanisms underlying this condition. We also find that some neuroimaging clues can be extremely relevant in the complex diagnostic process. A better understanding of the role of MFSD2A in brain physiology will provide new opportunities for on the development of targeted therapies or specific metabolic supplementation regimen aiming to treat neurological diseases. In particular, the identification and characterization of further patients harboring loss-of-function *MFSD2A* variants will support efforts to exploit LPCs as therapeutic lipids to improved DHA delivery and foster proper brain development in affected individuals.

Acknowledgments

The work was supported in part by National Research Foundation grants, Singapore NRF2016NRF-NRFI001-15 (to D.L.S.); by the Biomedical Research Council of A*STAR (to H. F.); as part of the Queen Square Genomics group at University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre.

Funding

The MRC (MR/S01165X/1, MR/S005021/1, G0601943), The National Institute for Health Research University College London Hospitals Biomedical Research Centre, Rosetree Trust, Ataxia UK, MSA Trust, Brain Research UK, Sparks GOSH Charity, Muscular Dystrophy UK (MDUK), Muscular Dystrophy Association (MDA USA).

REFERENCES

1. Nguyen LN, Ma D, Shui G, et al. Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature. 2014;509:503-506.
2. Chan JP, Wong BH, Chin CF, et al The lysolipid transporter Mfsd2a regulates lipogenesis in the developing brain. PLoS Biol. 2018;16:e2006443.
3. Alakbarzade V, Hameed A, Quek DQ, et al. A partially inactivating mutation in the sodium-dependent lysophosphatidylcholine transporter MFSD2A causes a non-lethal microcephaly syndrome. Nat Genet. 2015;47:814-817.
4. Guemez-Gamboa A, Nguyen LN, Yang H, et al. Inactivating mutations in MFSD2A, required for omega-3 fatty acid transport in brain, cause a lethal microcephaly syndrome. Nat Genet. 2015;47:809-813.
5. Guesnet P, Alessandri JM. Docosahexaenoic acid (DHA) and the developing central nervous system (CNS) - Implications for dietary recommendations. Biochimie. 2011;93:7-12.
6. Harel T, Quek DQY, Wong BH, et al. Homozygous mutation in MFSD2A, encoding a lysolipid transporter for docosahexanoic acid, is associated with microcephaly and hypomyelination. Neurogenetics. 2018;19:227-235.
7. Quek DQ, Nguyen LN, Fan H, Silver DL. Structural insights into the transport mechanism of the human sodium-dependent lysophosphatidylcholine transporter Mfsd2a. J Biol Chem. 2016;291:9383-94.

8. Riazuddin S, Hussain M, Razzaq A, et al. Exome sequencing of Pakistani consanguineous families identifies 30 novel candidate genes for recessive intellectual disability. Mol psychiatry. 2017;22:1604-1614.

9. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754-1760.

10. Van der Auwera GA, Carneiro MO, Hartl C, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics. 2013;43:11.10.1-11.10.33.

11. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38:e164.

12. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum Mutat. 2015;36:928-930.

13. Andreone BJ, Chow BW, Tata A, et al. Blood-Brain Barrier Permeability Is Regulated by Lipid Transport-Dependent Suppression of Caveolae-Mediated Transcytosis. Neuron. 2017;94:581-594.

14. Ahmad A, Moriguchi T, Salem N. Decrease in neuron size in docosahexaenoic acid-deficient brain. Pediatr Neurol. 2002;26:210-218.

15. Tesson C, Nawara M, Salih MA, et al. Alteration of fatty-acid-metabolizing enzymes affects mitochondrial form and function in hereditary spastic paraplegia. Am J Hum Genet. 2012;91:1051-1064.

16. Shaheen R, Maddirevula S, Ewida N, et al. Genomic and phenotypic delineation of congenital microcephaly. Genet Med. 2019;21:545-552.

17. Monies D, Abouelhoda M, Assoum M, et al. Lessons Learned from Large-Scale, First-Tier Clinical Exome Sequencing in a Highly Consanguineous Population. Am J Hum Genet. 2019;104:1182-1201.

LEGENDS

Fig. 1 Clinical characterization, neuroimaging features, genetic findings and predicted consequences of *MFSD2A* variants. (**a**) Pedigrees of the seven reported families. (**b**) Main clinical features include severe microcephaly, axial hypotonia, talipes equinovarus, and minor dysmorphic features (e.g., epicanthal folds and broad nasal bridge in patient 5). (**c**) Brain MRI of affected subjects performed at 3 years (Pt 1), 1 year (Pt 2), 17 years (Pt 3), 27 years (Pt 4), 2 months (Pt 5), 1 month (Pt 6), 2 years (Pt 7), and 4 months of age (Pt 8). First row: axial T2, FLAIR or T1-weighted images of the patients. Second row: corresponding sagittal T2 or T1-weighted images. There is severe microcephaly with mildly to severely simplified gyral pattern in all subjects. The cerebral white matter is reduced with consequent ventricular dilatation (asterisks), especially in patients 1, 2, 6, 7, and 8. The corpus callosum is barely visible and markedly short in Patients 1, 2, 6, 7, and 8 (empty arrows), while it is diffusely hypoplastic in Patient 5. Hypoplasia of the anterior portion of the corpus callosum is visible in patients 3 and 4 (arrows). Note that in all subjects the cingulate gyrus is present. The inferior vermian is small all subjects (arrowheads), with associated pontine hypoplasia in Patients 1, 2, 5, 6, 7, and 8. (**d**) Schematic drawing of *MFSD2A* with previously reported variants (in black) and the variants identified in this study (in red). Intragenic deletions are indicated by diagonal lines within the affected exon. (**e**) 3D structural models of the Mfsd2a variants. Positions of variants in the human Mfsd2a protein. Variants (cyan) were mapped to the published homology model of Mfsd2a (green). R326 is located at the putative extracellular gate and the R326H substitution

might disrupt gate closure. V250 and P164 are both located in helical bundles. Their substitution by larger amino acids (V250F and P164T) might perturb protein folding by steric clash with neighboring sidechains (e.g., W134, W118). P164T might also form a hydrogen bond with Y49 that is not seen in canonical Mfsd2a. Variants T198M and P493L are predicted to alter the local protein structure. (**f**) Percentage distribution of the main clinical features of *MFSD2A* patients. *DD* developmental delay; *ID* intellectual disability; *N/A* not applicable; *Pt* patient.

Fig. 2 Biochemical analysis of Mfsd2a variants. (**a**) Western blot probed for Mfsd2a and its mutants with β-actin used as loading control. (**b**) Confocal immunofluorescence micrographs of transiently transfected HEK293 cells with Mock, WT, D97A, P493L, T198M, P164T, R326H and V250F pathogenic variants showing Mfsd2a localization in green cell nuclei in blue (Hoechst stain), red arrows pointing to the cell surface localization of Mfsd2a and its mutants. (**c**) Titration of varying amounts of WT Mfsd2a DNA (μg) to normalize the expression levels to determine the amount of WT Mfsd2a needed for comparable expression levels with cells transfected with 2 mg of mutant construct DNA. (**d**) Transport of 50 μM ^{14}C LPC-DHA by comparable expression levels of MFSD2A in HEK293. Significance levels of difference compared with the transport activity of 0.1 μg of WT Mfsd2a (labeled WT on the graph) transport activity are labeled with asterisks; **** representing P value < 0.0001, *** representing P value <0.001, ** representing P value < 0.01, * representing P value <0.1.