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Functional assay for analysis of variants of uncertain significance in Maple Syrup Urine Disease

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INTRODUCTION

Enhanced molecular characterisation using high-throughput sequencing has significantly improved patient diagnosis and management. However, the identification of variants of uncertain significance (VUS) limits its use for clinical decision making, such as prenatal diagnosis in subsequent pregnancies. We describe the challenges in identifying intermediate Maple syrup urine disease (MSUD) by newborn screening, reinforcing the importance of functional studies to confirm variant pathogenicity in this era of molecular diagnostics.

MSUD is caused by a deficiency of branched-chain alpha-ketoacid dehydrogenase, responsible for degradation of branched chain amino acids (BCAA) leucine, isoleucine, and valine. Biallelic pathogenic variants in *BCKDHA*, *BCKDHB*, or *DBT* genes result in enzyme deficiency. Accumulation of leucine and alpha-ketoisocaproic acid result in metabolic intoxication characterised by developmental delay, encephalopathy, anorexia, feeding problems, and basal ganglia with movement disorders[1].

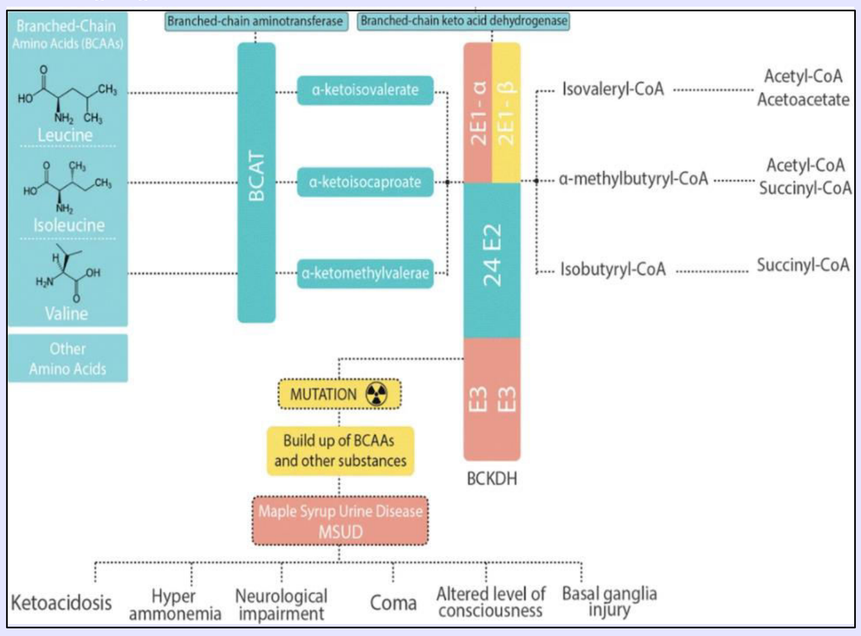


Figure 1: Schematic representation of the pathology of MSUD, depicting the catabolic pathway of BCAA[2]

CASE REPORT

A female infant presented with mild gross motor delay, aged 4 months and developed seizures with hypoglycaemia at 5 months. Plasma amino acid and urine organic acid findings were consistent with MSUD, despite a normal newborn screening test. Brain MRI showed bilateral symmetrical T2 hyperintense signal abnormalities involving white matter, globus pallidus, thalamus, brainstem, and dentate nuclei with restricted diffusion. Repeat MRI 10 months post-dietary-intervention showed the resolution of these changes and progression in myelination. Her clinical phenotype, including protein tolerance correlated with intermediate MSUD.

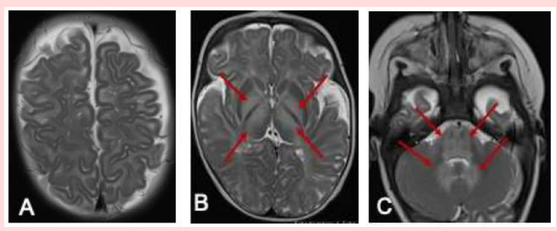


Figure 2a: MRI brain at 7 months
The red arrows show the hyperintense signal changes. T2-weighted axial images of an MRI scan of the brain at 7 months of age. **A–C**) show hyperintense signal changes in white matter (**A**), globus pallidus (**B**), and thalamus (**B**), cerebellar peduncle, and dentate nucleus (**C**).

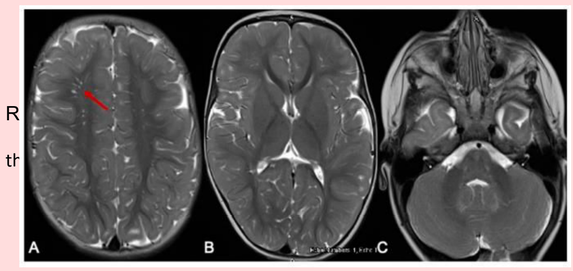


Figure 2b: Repeat neuroimaging 10 months later.
The red arrow indicates prominent perivascular spaces. T2- weighted axial images show significant improvement in myelination.
Prominent perivascular spaces are also seen (**A**-arrow).

previously noted signal changes of basal ganglia and

and the brainstem and dentate nuclei (**C**) observed.

METHODOLOGY

Plasma Amino Acids Analysis

Samples were deproteinised using s-sulphosalicylic acid precipitation, separated by centrifugation and derivatised with 6-aminoaquinolyl-N-hydroxysuccinimidyl carbamate. Derivatised amino acids were separated using a reverse phase BEH C18 column on a Waters UPLC system (Waters Corporation, MA, USA). The signal was detected by UV absorption at 260 nm and quantified by Empower software (Waters Corporation, Rydalmere, NSW, Australia).

Dried Blood Spot BCAA Analysis

Methanol-containing deuterated internal standards (leucine, isoleucine, and alloisoleucine) was used to measure BCAAs from punched 3 mm dried blood spots. The extract was dried and reconstituted with aqueous mobile phase (H2O with 0.1% formic acid and 0.02% heptafluorobutyric acid). A gradient elution using Waters Xevo UPLC system (Waters Corporation, Rydalmere, NSW, Australia) was used to separate the BCAA; with an aqueous mobile phase and organic mobile phase comprising acetonitrile with 0.1% formic acid and 0.02% heptafluorobutyric acid

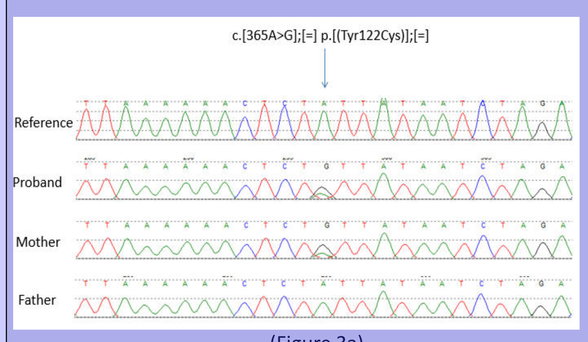
Leucine Decarboxylation Studies

Cultured skin fibroblasts were harvested at confluence. Cell pellets were resuspended in 0.2 M HEPES buffer containing Earle's salts and incubated with 1.85 MBq of [1-14C] leucine for 1, 2, and 3 h at 37 C. Duplicate samples were prepared with a control tracer (1.85 MBq of [1-14C] ornithine) to monitor sample viability, and heat-inactivated skin fibroblasts were included as reaction blanks. The 14CO2 released at a given incubation time point was trapped with 1 M KOH and subsequently determined using scintillation counting. The 14CO2 amount was extrapolated from a calibration curve relating the total radioactivity released against incubation time and expressed per microgram of cell protein.

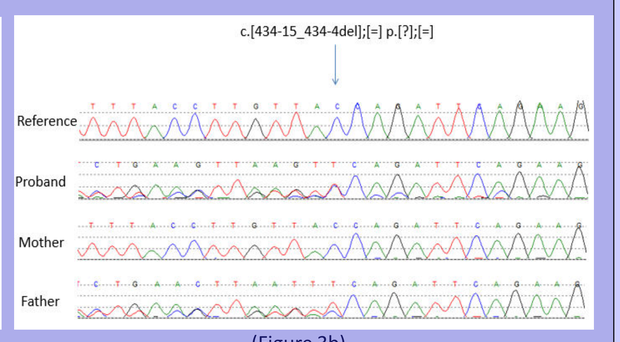
RESULTS

Molecular Analysis

Molecular analysis using next-generation sequencing identified two VUS, c.365A>G (p. Tyr122Cys) (Figure 3a) and c.434-15_434-4del (Figure 3b)in the *DBT* gene. Parental segregation studies confirmed the variants were in trans. The paternal c.434-15_434-4del variant was subsequently reclassified as pathogenic based on a previous report wherein it was detected as compound heterozygous in a patient with MSUD[3]. mRNA analysis revealed that this deletion arose due to an alternative acceptor splice site, which disrupted the open reading frame and may result in a null allele due to nonsense-mediated mRNA decay[4]. Criteria of evidence of pathogenicity considered as per the American College of Medical Genetics and Genomics (ACMG) guidelines were PVS1, PM2, PM3, and PP4moderate[5]. The maternal c.365A>G variant is reported in the ClinVar database as a VUS (ClinVar Variation ID: 166984; last accessed 28 July 2020).



(Figure 3a)



(Figure 3b)

Sanger sequence in the reverse direction shown in reverse complement. The forward direction is not possible due to a polymeric region upstream of the deletion.

LEUCINE DECARBOXYLATION STUDIES ON FIBROBLASTS

Functional testing in the form of [1-14C]- leucine decarboxylation studies was performed in cultured skin fibroblasts as orthogonal biochemical evidence to strengthen the likelihood of pathogenicity of this variant. Impaired decarboxylation was demonstrated, consistent with an intermediate form of MSUD (Table 1 below).

The maternal variant was subsequently reclassified as likely pathogenic based on the ACMG criteria of PM2, PM3, and PP4-moderate.

Cultured Skin Fibroblast Sample	$^{14}CO_2$ Released/hour/μg Protein	
	[1-^{14}C] Leucine	[1-^{14}C] Ornithine
Proband	2.1	31
Mother	28	14
Father	22	16
Normal controls	31, 50	23
MSUD affected	0.29	24

CONCLUSION

Advances in genomic sequencing technologies have resulted in the increasing discovery of novel disease-causing genes or rare variants, with the consequent need for orthogonal functional studies to validate their mutational impact and pathogenicity.

However, the availability of these types of functional studies for some of these rare inborn errors of metabolism has continued to decline with the increased utility of genetic testing.

An international collaborative effort may be warranted to preserve the capacity to perform some of these studies.

Leucine oxidation studies have been paramount in verifying the pathogenicity of compound heterozygous VUS and thus enabling reproductive family planning in this case.

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