Identification of Four New Mutations in Chilean Patients with Various Forms of Maple Syrup Urine Disease and Genotype-Phenotype Correlations

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Abstract

Background: Maple syrup urine disease (MSUD) is an autosomal recessive inherited metabolic disease caused by deficient activity of the branched-chain α-keto acid dehydrogenase (BCKD) enzymatic complex. BCKD is a mitochondrial complex encoded by four genes: BCKDHA, BCKDHB, DBT, and DLD. MSUD is predominantly caused by mutations in the BCKDHA, BCKDHB, and DBT genes encoding the E1α, E1β, and E2 subunits of the BCKD complex, respectively. The aim of this study was to characterize the genetic basis of MSUD by identifying point mutations in the BCKDHA, BCKDHB, and DBT genes in a cohort of Chilean MSUD patients, and to describe their phenotypic heterogeneity. It is a descriptive cross-sectional study with 18 MSUD patients was carried out using PCR and DNA sequencing.

Results: Four new pathogenic mutations were identified in the 18 patients that were analyzed: one in BCKDHA (p.Thr338Ile); two in BCKDHB (p.Gly336Ser e p.Pro240Thr); and one in DBT (p.Gly406Asp). Four additional pathogenic mutations found in the analyzed patients have been described previously.

Conclusion: There were no correlations observed between genotypes and phenotypes. Thus, if MSUD is diagnosed more promptly, such as in neonatal screening, it might be possible to establish genotype-phenotype relationships more efficiently. Keywords: inborn errors of metabolism; maple syrup urine disease; branched-chain amino acids; valine; leucine; isoleucine.

Background

Maple syrup urine disease (MSUD) (OMIM #24860), also known as Leucinose, is an inborn error of metabolism (IEM) caused by deficiency of the branched-chain keto acids dehydrogenase (BCKD) complex activity, causing accumulation of branched-chain amino acids (BCAA), leucine (LEU), isoleucine (ILE) and valine (VAL), and their keto acids (BCKA). The BCKD complex is a multienzyme macromolecule with three catalytic components (E1, E2, E3) (1). Deficiency of this complex is responsible for increases in the branched chain amino acids leucine, valine and isoleucine in physiological fluids, as well as their related α-keto acids (2). The accumulation of these amino acids mainly affects the central nervous system (CNS) (3). Studies suggest the occurrence of oxidative stress in patients with this disease and in those undergoing treatment (4).

Many mutations that cause MSUD have already been described involving the catalytic subunits of the branched chain α-keto acid dehydrogenase (BCKD) complex, and, according to altered loci, three genetic subtypes have been proposed: type Ia (MIM # 608348) for mutations found in BCKDHA (subunit E1α), type Ib (MIM #248.611) involving mutations found in BCKDHB (subunit E1β), and type II (MIM # 248610), with mutations in the DBT gene (subunit E2) (5,6). BCKDHA has been mapped to human chromosome 19, at 19q13.2. The gene encompasses approximately 27.2 kb of DNA, with coding sequence distributed as 9 exons and involving 1791 bp. BCKDHB is situated at 6q14.1, spans approximately 240 kb of genomic DNA, and has 11 exons encoding 1572 bp. DBT, at 1p31, has 63 kb of DNA, also has 11 exons and coding sequence of 10831 pb (3,7). Considering these three determinate disease genes, more than 140 mutations are known, and have already been described in the literature. (8).

MSUD is inherited in a recessive autosomal fashion, and its global incidence is estimated at 1 in 185,000 newborns. Although it is a rare defect, in some Mennonite populations settled in Pennsylvania, and in some cities of the United States, the estimated elevated incidence is 1 in 200 live births (3). A study carried out in Portugal by Quental et al. (2010), based on an analysis of cases diagnosed by mass spectrometry, found an incidence of 1 per 86,800 newborns (9). In Brazil, a study conducted by Margutti (2015) identified 11 new mutations in 25 patients with the disease, with three in the BCKDHA gene (p. Pro39Leu, p. Gly56Arg, and p. Tyr120Ter), six in BCKDHB (p. Arg63Pro, p. Gly131Val, p. Glu146Gln, p.Phe149Cys, p. Cys207Phe, and p. Lys211Asn) and two in DBT (p. Glu148Ter and p. Glu417Val) (10).

The clinical manifestations of patients with MSUD are varied, and depend on the levels of residual enzyme activity. The clinical phenotypes associated are classified as classic, intermediate, intermittent, responsive to thiamine therapy, and lipoamida dehydrogenase deficiency (E3 subunit), depending on, among others, the age of onset and severity of the disease. In the classical form of MSUD, with less than 3% residual enzyme activity, symptoms appear soon after birth. Patients typically have ketosis and plasma concentrations of LEU over 2,000 μmol/l. In untreated newborns, maple syrup odor can be detected in earwax within the first 12-24 h, and in urine 48-72 h after birth. Elevated plasma concentrations of BCAA, as well as widespread disturbances to plasma concentrations of amino acids are present at 12-24 h of age; elevation of keto acids and ketonuria, irritability, are observed 24-72 hours after delivery; encephalopathy manifesting as lethargy and intermittent breathing difficulties are seen after 4 to 5 days of life; and coma and central respiratory failure can occur between 7 to 10 days (1).

The intermediate form of MSUD features in infancy and childhood, being characterized by psychomotor developmental delay, failure to thrive, seizures and walking difficulty. Ketosis and plasma concentrations of LEU less than 2,000 µmol/l are typical. Although BCAA elevation is persistent, and there is neurological impairment, it is not seen in severe newborn organ decompensation of the classical form. Enzymatic activity in these cases is between 3 and 30% (2).

The intermittent form appears during infancy or childhood, and the patient usually has normal growth and development, with, bouts of ataxia that may be accompanied by ketoacidosis. The increase in BCAA only happens during decompensations.

The sensitivity to thiamine form has a clinical presentation similar to intermediate or intermittent cases, without acute decompensation. Thiamine is a subunit E1 cofactor, regulating the activity of all of the enzyme complex. Thus, the administration of thiamine decreases serum levels of BCAA. The doses of thiamine used may vary from 10 to 1,000 mg per day (11).

The E3 subunit deficiency form is very rare, having been reported in approximately 20 cases from around the world. The prognosis for this form of the disease seems to be associated with residual enzymatic activity between 0% and 25% (11).

There are no literature studies that describe genotypic profiles of MSUD patients in the Chilean population.

Objectives

We aimed to identify mutations in the genes BCKDHA, BCKDHB and DBT in a cohort of Chilean patients clinically diagnosed with MSUD, and to analyze the clinical characteristics of patients, in order to identify possible genotype-phenotype correlations.

Methods

Patients

The nutrition team from the Genetics and Metabolic Diseases Laboratory of the Nutrition and Food Technology Institute, Dr. Fernando Monckenberg Barros, Chile University (INTA), have established an agreement with Porto Alegre Clinical Hospital, Brazil, to perform collaborative work on a molecular study of mutations in Chilean patients with MSUD.

From June to August 2012, DNA was extracted from 5-10 mL blood samples of 36 patients who agreed to participate in the research.

Porto Alegre Clinical Hospital, through the Brazilian MSUD Assistance and Research Network, shared these samples in order to have the three main genes involved in MSUD analyzed at the molecular level. To date, DNA from 18 patients has been analyzed.

Molecular Analysis

DNA was extracted from peripheral blood leukocytes for the molecular analysis of the three genes involved in MSUD (BCKDHA, BCKDHB, and DBT).

DNA Sequencing

PCR-amplified fragments were sequenced on an ABI 3500xL Genetic Analyzer capillary sequencer platform, a 24-capillary DNA analysis system (Applied Biosystems, Foster City, CA, USA) using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Mutation Analysis

Sequencing results were visualized using FinchTV® version 1.4.0 software (Geospiza, Seattle, WA, USA) and compared with the relevant reference sequences from the GenBank® database (12). The nomenclature for the description of sequence variants detected was derived from the recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen) (13).

In order to verify the pathogenic potential of missense mutations, in silico analysis was performed using MutPred® v1.2 (14), Polyphen-2®-Polymorphism Phenotyping v2 software, and SIFT® (15). Sequence variants were also evaluated for their disease-causing potential using the Mutation Taster application (16).

Clinical Data

The nutrition team from INTA, Chile University, provided clinical data regarding the patients studied. The clinical data provided were anthropometry at birth, complications during pregnancy, birth date, age of diagnosis, hospitalization, and clinical and laboratory tests for leucine, valine and isoleucine.

Statistical Analysis

Fisher's exact test was applied in a sample of patients with the more prevalent mutation, p.Ile214K, in an attempt to assess the degree of correlation between clinical and genetic variables, and to check the possibility of establishing genotype/phenotype relationships. Fisher's exact test is a statistical significance test generally used in contingency table analysis of small sample sizes. The probability of significance was adopted with p-values < 0.05.

Results

Molecular Analysis

Of the 18 patients 88% presented with mutations in the BCKDHB gene, 1 patient had a mutation in the gene BCKDHA, and 1 patient harbored a mutation in DBT. A total of eight mutations were found in the samples, and four of these (50%) were new, previously uncharacterized mutations. The new mutations found were c.[1006 G>A] and c.[718 C>A], in BCKDHB, c.[1013 C>T] in BCKDHA, and c.[1217 G>A] in DBT (Table 1).

This study was able to identify regions with the highest incidence of mutations in exon 6 of BCDKHB. Exons 4, 5, 9 and 10 exhibited 1 mutation in each, as depicted in the schematic representation of BCKDHB in Figure 1.

Table 1 – Pathogenic variants detected in BCKDHA, BCKDHB and DBT genes of Chilean MSUD patients.

Patient	Gene	Nucleotide	Protein Prediction
1	**BCKDHA**	**c.[1013 C>T] §+[1013 C>T] §**	**p.Thr338Ile + Thr338Ile**
2	BCKDHB	c[.595_596delAG] +[641 T>A]	p. Pro200Stop +Ile214Lys
3	**BCKDHB**	**c.[641 T>A] + [1006 G>A]§**	**p.Ile214Lys + Gly336S**
4	BCKDHB	c.[595_596delAG] +[641 T>A]	p.Pro200Stop +Ile214Lys
5	BCKDHB	c.[641 T>A] + [641 T>A]	p.Ile214KLys+Ile214Lys
6	**DBT**	**c.[1217 G>A]§+ c.[1217 G>A]§**	**p.Gly406Asp+Gly406Asp**
7	BCKDHB	c.[595_596delAG]+ [641 T>A]	p. Pro200Stop + Ile214Lys
8	**BCKDHB**	**c.[718 C>A]§ + [718 C>A]§**	**p.Pro240Thr+ Pro240Thr**
9	BCKDHB	c.[641 T>A] + [641 T>A]	p.Ile214KLys+Ile214Lys
10	BCKDHB	c.[392 G>T] +[595_596delAG]	p.Gly131Val + Pro200Stop
11	BCKDHB	c.[641 T>A] + [641 T>A]	p.Ile214KLys+Ile214Lys
12	BCKDHB	c.[641 T>A] + [641 T>A]	p.Ile214KLys+Ile214Lys
13	BCKDHB	c.[392 G>T] + [392 G>T]	p.Gly131Val+ Gly131Val
14	BCKDHB	c[.595_596delAG]+ [641 T>A]	p. Pro200Stop +Ile214Lys
15	**BCKDHB**	**c.[718 C>A]§ + [718 C>A]§**	**p.Pro240Thr+ Pro240Thr**
16	BCKDHB	c[.595_596delAG]+ [641 T>A]	p. Pro200Stop +Ile214Lys
17	BCKDHB	c.[1067 C>T] + [1067 C>T]	p.Pro356Leu + Pro356Leu
18	BCKDHB	c.[641 T>A] + [641 T>A]	p.Ile214KLys+Ile214Lys

- New Mutations

Figure 1 – Schematic representation of BCKDHB and mutations; the new mutations are presented in bold. black lines with numbers: coding exons; black horizontal lines: introns; untranslated regions (5' and 3' UTR)

Mutation Pathogenicity

Among the mutations already described in the literature, the Ile214Lys mutation of Spanish origin had the highest incidence, totaling 77 % of the patients, followed by mutation Pro200*, also of Spanish origin in 33% (Table 2). Of these mutations, those found in the heterozygous state were p.Gly131Val, p.Pro200Stop, and p.Ile214Lys. Homozygous mutations identified were p.Gly131Val, and p.Ile214lys.

Table 2 – Mutations already described in the literature found in Chilean MSUD patients.

	Gene	Nucleotide	Protein Prediction	Type	Origin	Phenotype	Reference	Frequency in the sample
Exon 4	BCKDHB	c.[392 G>T]	p.Gly131Val	M	NA	NA	Margutti AV, 2015	2/18
Exon 5	BCKDHB	c. [595_596delAG]	p.Pro200Stop	D	Spanish	Classic	Henneke M, 2003	6/18
Exon 6	BCKDHB	c.[641 T>A]	p.Ile214Lys	M	Spanish	Classic	Rodriguez-Pombo,2006	14/18
Exon 10	BCKDHB	c.[1067 C>T]	p.Pro356Leu	M	Portuguese	Classic	Quental, 2008	1/18

NA: Not Available.

M: Missense.

D: Deletion.

The new mutation Pro240Thr was found in 16% of the samples; it was also located in exon 6 of BCKDHB, and was the most prevalent of the new mutations. After being subjected to in silico analysis, the new mutations were all considered pathogenic (Table 3). Of these new mutations, the ones found in the homozygous state were p.Thr338Ile, p.Gly406Asp and p.Pro240Thr. Those found as heterozygous were p.Pro240Thr and p.Gly336Ser.

Table 3 – New mutations found in Chilean MSUD patients.

Region	Gene	Nucleotide	Protein Prediction	Type	Pathogenicity			Classification	Frequency in the sample
					Sift®	Polyphen-2®	Multipred®		
Exon 8	BCKDHA	c. [1013C>T]	p.Thr338Ile	M	Pathogenic	Pathogenic	Pathogenic	Pathogenic	1/18
Exon 6	BCKDHB	c.[718C>A]	p.Pro240Thr	M	Pathogenic	Pathogenic	Pathogenic	Pathogenic	3/18
Exon 9	BCKDHB	c. [1006G>A]	p.Gly336Ser	M	Pathogenic	Pathogenic	Pathogenic	Pathogenic	1/18
Exon 10	DBT	c. [1217G>A]	p.Gly406Asp	M	Pathogenic	Pathogenic	Pathogenic	Pathogenic	1/18

M: Missense.

Clinical Analisis

Anthropometric Assessment

According to clinical data, patient 6 was born weighing 4 kg, which is characterized as macrosomia, common in newborns of pregnant women who have gestational diabetes, which was the case in this child. Patient number 10 was born with insufficient weight, but her mother had no problems during pregnancy. The mother of child number 14 presented with preeclampsia during pregnancy, and this patient was born with low weight, also a consequence of complications in pregnancy. Seventy two percent (72%) of the children were

born with weight and lengths appropriate for their gestational ages, according to the classification of Intergrowth 21st, as seen in Table 4.

Table 4 – Anthropometric data of patients at birth.

Patient	Birth Weight (g)	Birth Length (cm)	Nutritional Diagnostic	Pregnancy Complications
1	3600	51	AGA	no
2	2825	47,5	AGA	Anemia
3	2820	48	AGA	no
4	3290	51,5	AGA	no
5	2835	50	AGA	no
6	4000	43	LGA	Gestational Diabetes/ UTI
7	3240	48	AGA	no
8	2830	49	AGA	Hepatic Cholestasis
9	3150	50	AGA	no
10	2600	47	SGA	no
11	3900	52	AGA	no
12	3890	51	AGA	Hydronephros
13	3050	48	AGA	no
14	1580	42	SGA	Preeclampsia
15	3300	50	AGA	no
16	2940	49	AGA	no
17	3000	50	AGA	no
18	2750	48	AGA	no

 Source: Villar et al. (2014).

UTI: urinary tract infection.

SGA: small for gestational age.

AGA: appropriate for gestational age.

LGA: large for gestational age

Clinical Evaluation

According to the age of onset of symptoms, 95% of the patients in this cohort were considered to have the classic form of the disease, and 5% with intermediate form. The age at diagnosis ranged from 9 days to 7 months. Leucine levels ranged from 440 to 3962 µmol/L at diagnosis (normal range 35 - 217 µmol/L). Neuropsychomotor Developmental Delay (NPMDD) occurred in 13 of the 18 children studied, and 4 had a mild degree, 3 a moderate degree, and 6 had severe DNPMD. Only 2 children did not have NPMDD, and that information was not available for 3. Therefore, in children for whom NPMDD data were available, the majority, 40%, had a serious degree of delayed neuropsychomotor development.

Table 5 – Characterization of Chilean MSUD patients (n = 18) at INTA.

Patient	Diagnostic Time (days)	Leucine (µmol/L)	NPMDD	First Hospitalization (days)	Presentation according to diagnostic time
1	10	741	severe	10	Classic
2	9	750	severe	10	Classic
3	14	3560	moderate	7	Classic
4	13	2638	light	32	Classic
5	22	2600	light	30	Classic
6	11	750	NA	48	Classic
7	30	1640	light	5	Classic
8	8	3962	severe	2	Classic
9	21	2000	severe	30	Classic
10	9	993	moderate/severe	20	Classic
11	9	440	severe	4	Classic
12	16	1027	no	32	Classic
13	17	1090	NA	36	Classic
14	30	1716	no	10	Classic
15	210	1653	light	90	Intermediate
16	17	1467	moderate	14	Classic
17	17	1038	severe	NA	Classic
18	27	2075	NA	10	Classic

NA: Not Available.

NPMDD: Neuropsychomotor Developmental Delay

The biochemical tests values at time of diagnosis provided by INTA were quite high. Leucine levels ranged from 440 to 3962 µmol/L (normal range 35-270 µmol/L), valine ranged from 133 to 1464 µmol/L (normal range 51-325 µmol/L), and isoleucine ranged from 38 to 759 µmol/L (normal range 13-135 µmol /L), as shown in Table 6.

Table 6 – Laboratory values at diagnosis of Chilean MSUD patients at INTA.

Pacient	Diagnostic time (days)	Leucine (35-270 µmol/L)	Valine (51-325 µmol/L)	Isoleucine (13-135 µmol/L)
1	10	741	759	402
2	9	750	512	50
3	14	3560	710	512
4	13	2638	585	372
5	22	2600	1180	730
6	11	750	726	657
7	30	1640	174	53
8	8	3962	277	215
9	21	2000	369	190
10	9	993	838	294
11	9	440	1464	759
12	16	1027	117	176
13	17	1090	480	290
14	30	1716	215	525
15	210	1653	1171	586
16	17	1467	466	419
17	17	1038	133	38
18	27	2075	726	170

According to the signs and symptoms presented by the patients, the most prevalent were axial hypotonia, identified in 83% of the children, followed by NPMDD in 77% of the cases, intellectual deficit in 61%, and food intolerance and urine with characteristic odor in 55%. Strabismus, pyramidal syndrome, and encephalopathy were reported in 44% of patients, seizures in 38%, macrocephaly and need for mechanical ventilation in 27%, gastrostomy and ataxia in 22%, apnea and mucous and skin lesions in 16%, attention deficit disorder and hyperactivity in 14%, extrapyramidal syndrome in 11%, and coma in 5% (table 7).

Table 7 – Signs and symptoms presented by Chilean MSUD patients (n = 18) of INTA.

Signs and Symptoms	Prevalence
Axial hypotonia	83%
NPMDD	77%
Intellectual deficit	61%
Urine with maple syrup odor	55%
Food intolerance	55%
Strabismus	44%
Encephalopathy	44%
Pyramidal syndrome	44%
Seizures	38%
Mechanical ventilation	27%
Macrocephaly	27%
Ataxia	22%
Gastrostomy	22%
Apnea	16%
Mucous and skin lesions	16%
ADDH	14%
Extrapyramidal syndrome	11%
Coma	5%

NPMDD: Neuropsychomotor Developmental Delay

ADDH: Attention deficit disorder and hyperactivity.

Genotype-Phenotype Correlations

INTA staff ranked the phenotypes of their patients, based on age at diagnosis. Intermittent and sensitive to thiamine phenotypes were not found in this sample. However one patient was classified with an intermediate form of MSUD, with diagnosis done at 90 days of age, and 17 patients with the classical form, with diagnosis made at 9 to 30 days of life (Table 8). The clinical situation of these patients varied, exhibiting NPMDD and poor prognosis, leading in some cases to death. Among patients with the classical phenotype, four died, six had severe NPMDD, two had moderate NPMDD, and two were diagnosed as light NPMDD. In the patient with the intermediate phenotype the NPMDD was light.

Leucine level values were highly variable. Even in patients with classical presentation of the disease, these values ranged from 741 µmol/L to 3962 µmol/L, as shown in Chart 1 and in Table 8.

Table 8 – Genotype-phenotype correlations of Chilean MSUD patients (n = 18).

Patient	Birth year	First hospitalization (days)	Diagnosis Time (days)	LEU (µmol/L)	Clinical Situation	Phenotype	Genotype
1	2002	10	10	741	Severe	Classic	c.[1013 C>T][§] +[1013 C>T] [§]
2	2002	10	9	750	Severe	Classic	c[.595_596delAG] + c.[641T>A]
3	1995	7	14	3560	Moderate	Classic	c.[641 T>A] + [1006 G>A][§]
4	2006	32	13	2638	Light	Classic	c.[595_596delAG] +c.[641T>A]
5	2012	30	22	2600	Light	Classic	c.[641 T>A] + [641 T>A]
6	2001	48	11	750	Death	Classic	c.[1217 G>A][§]+ c.[1217 G>A][¶]
7	2004	5	30	1640	Death	Classic	c.[595_596delAG]+c.[641T>A]
8	2004	2	8	3962	Severe	Classic	c.[718 C>A][§] + [718 C>A][§]
9	2003	30	21	2000	Severe	Classic	c.[641 T>A] + [641 T>A]
10	1997	20	9	993	Moderate/Severe	Classic	c.[392 G>T] + [595_596delAG]
11	2006	4	9	440	Severe	Classic	c.[641 T>A] + [641 T>A]
12	1997	32	16	1027	NA	Classic	c.[641 T>A] + [641 T>A]
13	1998	36	17	1090	Death	Classic	c.[392 G>T] + [392 G>T]
14	2003	10	30	1716	NA	Classic	c[.595_596delAG]+ [641T>A]
15	1999	90	210	1653	Light	Intermediate	c.[718 C>A][§] + [718 C>A][§]
16	1996	14	17	1467	Moderate	Classic	c[.595_596delAG]+ [641 T>A]
17	2000	ND	17	1038	Death	Classic	c.[1067 C>T] + [1067 C>T]
18	2002	10	27	2075	NA	Classic	c.[641 T>A] + [641 T>A]

NA: Not Available.

NPMDD: Neuropsychomotor Developmental Delay.

- : New mutation.

In an attempt to establish correlations between genotype and phenotype, it was observed that among the mutations found in the homozygous state, the c. [1013 C>T] or p.Thr338Ile (new), c. [1217 G>A] or p.Gly406Asp (new), c. [392 G>T] or p.Gly131Val, c. [1067

C>T] or p.Pro357Leu, and c. [641 T>A] or p.Ile214Lys expressed the classic form in 100% of the patients. The new mutation c. [718 C>A] or p.Pro240Thr, when homozygous, was associated with the intermediate form of the disease in 50% of cases. All heterozygous mutations were found in patients with classic phenotypes (Chart 1).

Chart 1 – Representation of leucine values in µmol/L at diagnosis in patients classified with the classic phenotype, according to age at diagnosis.

Also, a representative chart of the correlation between leucine values and diagnostic age was drawn. Leucine values ranged very heterogeneously according to age at MSUD diagnosis; hence, it was not possible to identify any correlations among them (Chart 2).

Chart 2 – Correlation between leucine values in µmol/L and age at diagnosis in days, with a p value of 0.989.

Once the p.Ile214Lys mutation was identified in 11 of the 18 samples, it was possible to combine clinical information regarding these patients in an attempt to establish genotype/phenotype correlations. The following were assessed by Fisher`s exact test: correlation between the p.Ile214Lys mutation and the degree of NPMDD, the mutation and the classic and intermediate phenotypes, the allele type (hetero- and homozygous), and the severity of NPMDD, and the allele type and the classic and intermediate phenotypes. None of the correlations were associated with a probability value of significance smaller than 5%. Thus we cannot state that there is any correlation between these variables in this sampling (Table 9).

Table 9 – Associations between patients with the p.Ile214Lys mutation and the type of allele.

Mutation	NPMDD		Total	P-Value
	Light/Moderate	Severe/Death		
Others	1	6	7	0.282
Ile214Lys	4	4	8	

Mutation	Phenotype		Total	P-Value
	Intermediate	Classic		
Others	1	6	7	0.245
Ile214Lys	0	11	11	

Allele	NPMDD		Total	P-Value
	Light/Moderate	Severe/Death		
Heterozygous	3	2	5	0.394
Homozygous	1	2	3	

Allele	Phenotype		Total	P-Value
	Intermediate	Classic		
Heterozygous	0	6	6	0.245
Homozygous	0	5	5	

p-value<0,05

NPMDD: Neuropsychomotor Developmental Delay

Others: mutations identified in this study other than I214K.

Discussion

In this study, 5% of patients presented with the intermediate form of MSUD, and the majority, 95 percent, had the classic form, mainly due to early onset of symptoms, with prevalence of homozygous mutations observed in 61% of cases. It is interesting that in none of the patients had consanguinity been reported involving the parents, and in two cases there were historical reports of illness in the family. This fact might be linked to greater genetic homogeneity in the Chilean population. MSUD is initially diagnosed through clinical examinations, and by finding the peculiar odor of maple syrup in urine. BCAA serum analysis is the most convenient method, primarily in neonatal screening. If a patient has high dosages of leucine, valine, isoleucine, or alloisoleucine (>5 mm/L), MSUD must be considered (17). In addition, levels of leucine greater than 1,000 μmol/L are considered critical, as they can produce irrevocable damage, or even lead to death (18). Levels of leucine at diagnosis in our study participants ranged from 440 to 3962 μmol/L, and most children (72%) presented with more than 1,000 μmol/L. BCKDHB harbored the great majority of mutations, 6 of the 8 mutations found in this cohort are located on the E1b subunit. Among the mutations already described in the literature, Ile214Lys was the one here with the highest incidence, totaling 77% of the patients. The new mutation with high incidence was Pro240Thr, found in 16% of samples, and also located in BCKDHB exon 6.

According to Rodríguez-Pombo and collaborators (2006), the p.Ile214Lys mutation is responsible for endangering the stability of the coding protein causing a classic form of the disease. The local source of this variant is the region of Chile colonized by the Spanish. In our findings, we observed a certain spectrum of symptoms in patients with this mutation. Here, patients 5, 9, 11, 12, and 18 had the p.Ile214Lys mutation in the homozygous state, while children 9 and 11 presented with serious NPMDD. Child 5 had a light manifestation, and data was unavailable for patients 12 and.

Other factors to consider are the level of leucine and the evolution of the disease. It was observed that patient 5, with a level of leucine of 2600 μmol/L at diagnosis, showed only light NPMDD, while patient 11, with a leucine level of 440 μmol/L had serious NPMDD. Therefore, we found no association between initial levels of leucine and the severity of MSUD. Patients 11 and 12 are siblings, and have homozygous p.Ile214Lys mutations. However, the initial values of leucine levels were very different. In addition, patient 12 is the eldest, born in 1997; it took 90 days to establish his MSUD diagnosis, while the younger brother, born in 2006, already with a history of illness in the family, obtained a diagnosis in much shorter time - 9 days. The technology for the diagnosis of advanced disease, as well as genetic counseling that this family already undergone, influenced the speed of diagnosis, early treatment, and of course, in disease evolution.

This fact can also be verified with the p.Pro240Thr variant, a new mutation found, which, when homozygous, manifested in patient 8 as serious NPMDD, and also in patient 15 with light NPMDD.

High levels of leucine are seen at diagnosis in most critical patients, and this can produce permanent damage or even death (19). The results of a Brazilian study (18) on the spectrum of MSUD over the last two decades found no significant association between the severity of mental development delay and levels of leucine at the time of diagnosis, which is consistent with our observations. This can be attributed to the fact that long-term metabolic control can be considered a determining factor more important in cognitive and psychomotor development than initial levels of leucine (19).

It eventuates that, considering age at diagnosis of MSUD, among the new mutations, p.Thr338Ile, p.Gly336Ser, and p.Gly406Asp feature a classic manifestation, with early onset of symptoms. Only p.Pro240Thr exhibited onset of symptoms later in one patient, favoring an intermediate phenotype. However, one of the major problems observed is the time between onset of symptoms and the time duration to disease diagnosis in locations without neonatal screening for MSUD, as is the case in Chile. One of our patients with the p.Pro240Thr mutation in the homozygous state, who was considered to exhibit an intermediate phenotype at age of diagnosis, remained in hospital for 90 days. His diagnosis was at 210 days, but how much time passed between onset of symptoms and that diagnosis is unknown. It is worth noting, as discussed before, that biochemical values of BCAA and clinical evolution in this patient was not consistent with that described in the literature for the intermediate phenotypes. Thus, a patient's clinical situation is not believed to be the best way of correlating phenotype and genotype, because neurological deterioration is directly associated with the absence of early diagnosis and, therefore, with the absence of implementation of adequate nutritional treatment, which is fundamental to the control of serious levels of BCAA and its metabolites (metabolic control). In a study by Morton et al. (2002), in which patients had access to metabolic formula, and where a clinical protocol was followed in the acute early stage of the disease, the overall results were better, and patients reached more appropriate developmental stages (20).

Although some patients were diagnosed in the first month of life, it is known that the time between diagnosis and receipt of metabolic formula can be long and variable.

Thus, if MSUD was diagnosed more rapidly by neonatal screening, perhaps it would be possible to establish genotype-phenotype associations more efficiently. MSUD meets most of the criteria of Wilson and Jungner (1968) for neonatal screening (21). In countries where MSUD is included in neonatal screening, patients are usually diagnosed before the 10th day of life. On the other hand, in countries where it is not included in public neonatal screening programs, such as Chile, diagnosis is usually delayed.

Early diagnosis and treatment in MSUD are crucial to reduce the severity of brain damage (1).

MSUD treatment includes diets low in protein, supplementation with BCAA-free formula, and symptomatic treatment during metabolic crises, such as ingestion of mannitol to treat brain edema, injection of insulin to lower blood glucose, and use of N-carbamylglutamate to reduce serum levels of ammonia (22).

As the liver is responsible for 15% of the production of BCKD, liver transplantation can restore enzyme activity in patients with MSUD (23). Liver transplantation may benefit patients with the classical form; however, it does not reverse the disease process. (24).

Prenatal diagnosis is important to identify defects before birth, especially with difficult to treat conditions. Accurate genetic analysis of probands has allowed DNA-based prenatal diagnosis of single gene disorders. You et al. (2014) reported a case of a Chinese family with MSUD, with BCKDHA gene mutations; prenatal diagnosis for the fetus was performed (25). In a study by Li et al. (2015), prenatal diagnosis for a mutation in BCKDHB was also made (26). In both cases, treatment was started at birth, and diagnosis was possible due to awareness of an existing mutation in the family.

Conclusions

In this study, we found a total of eight mutations, of which four are not described in the literature: p.Thr338Ile, p.Gly336Ser, p.Gly406Asp, and p.Pro240Thr. Six of the eight identified mutations are located in exon 6 of BCKDHB, and this gene was the locus that presented the most frequent mutations in this population, with the I214K variant present in 77% of the patients.

Considering the age at disease diagnosis, among the new mutations, patients with p.Thr338Ile, p.Gly336Ser, and p.Gly406Asp exhibited a classic manifestation, with early onset of symptoms: only p.Pro240Thr presented diagnosis at a later time, manifesting as an intermediate phenotype in one patient. The biochemical values for BCAA levels and clinical evolution in those patients were not consistent with those described in the literature for these clinical phenotypes.

We found no association between initial levels of leucine and the severity of MSUD. This can be attributed to the fact that long-term metabolic control can be considered a determining factor that is more important in cognitive and psychomotor development than initial levels of leucine.

If MSUD were to be diagnosed more promptly by neonatal screening, perhaps it would be possible to establish genotype-phenotype associations more efficiently.

Abbreviations

MSUD: Maple syrup urine disease

BCKD: Branched-chain α-keto acid dehydrogenase

BCKDHA: Branched chain keto acid dehydrogenase E1, alpha polypeptide

BCKDHB: Branched chain keto acid dehydrogenase E1, beta polypeptide

DBT: Dihydrolipoamide branched chain transacylase E2

DLD: Dihydrolipoamide dehydrogenase

IEM : Inborn Error of Metabolism

BCAA: Branched-chain amino acids

LEU: Leucine

ILE: Isoleucine

VAL: Valine

BCKA: Branched-chain keto acids

DNA: Desoxirribonucleic acid

CNS: Central nervous system

INTA: Nutrition and Food Technology Institute, Dr. Fernando Monckenberg Barros, Chile University

PCR: Polimerase Chain Reaction

NPMDD: Neuropsychomotor Developmental Delay

Declarations

Ethics approval and consent to participate

This research was approved by Ribeirão Medical School Research Ethics Committee and by the National Commission of Ethics in Research, committee's reference number 2.252.930 and it is in accordance with the Helsinki Declaration of 1975. Parents/guardians signed an Informed Consent form giving permission for participation in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]. For more information, the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DRRC was the Phd studant who performed the participants biomolecular study and analysed the correletions between phenotype and genotype, ,AVBM standardized this study methodology and helped with the mutations analysis , WASJ helped with the mutations analysis ,DFG helped with the methodology standardization , GAM helped the biomolecular study, AAM did the sequencing reaction, IVDS helped with the mutations analysis , VC, VH and GC were responsable for colecting the blood samples and all the participants clinical data, ESB helped with biomolecular study and , JSCM was DRRC advisor and helped with the biomolecular analysis and the clinical data interpertation for the genotype/phenotype correlation. All authors read and approved the final manuscript.

References

1. Strauss K, Puffenberger E, Morton D. Maple syrup urine disease. GeneReviews. 2006;30:1993–2013.

2. Chuang JL, Wynn RM, Moss CC, Song JLJL, Awad N, H. Mandel DT. Chuang, Structural and biochemical basis for novel mutations in homozygous Israeli maple syrup urine disease patients: a proposed mechanism for the thiamin-responsive phenotype. J Biol Chem. 2004;279:17792–17800.

3. Chuang D, Shih V. Maple syrup urine disease (Branched-chain ketoaciduria). In: Scriver C, Beaudet A, Sly W, Valle D, editors. The metabolic and molecular bases of inherited disease. 8th ed. Mcgraw-Hill; 2001. p. 1971–95.

4. Barschak AG, Sitta A, Deon M, Barden AT, Dutra-Filho CS, Wajner M, et al. Oxidative stress in plasma from maple syrup urine disease patients during treatment. Metab Brain Dis. 2008;23:71–80.

5. Fisher CR, Fisher CW, Chuang DT, Cox RP. Occurrence of a Tyr393—Asn (Y393N) mutation in the E1 alpha gene of the branched-chain alpha-keto acid dehydrogenase complex in maple syrup urine disease patients from a Mennonite population. Am J Hum Genet. 1991;

6. Rodríguez-Pombo P, Navarrete R, Merinero B, Gómez-Puertas P, Ugarte M. Mutational spectrum of maple syrup urine disease in Spain. Hum Mutat. 2006;

7. McKusick VA. Mendelian Inheritance in Man and its online version, OMIM. Am J Hum Genet. 2007;80:588–604.

8. Stenson PD, Mort M, Ball E V., Shaw K, Phillips AD, Cooper DN. The Human Gene Mutation Database: Building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Human Genetics. 2014.

9. Quental S, Vilarinho L, Martins E, Teles EL, Rodrigues E, Diogo L, et al. Incidence of maple syrup urine disease in Portugal. Mol Genet Metab. 2010;100:385–7.

10. Margutti A. Perfil genotípico de pacientes brasileiros com doença da urina de xarope de bordo. Faculdade de Medicina de Ribeirão Preto/Universidade de São paulo; 2015.

11. Mitsubuchi H, Owada M, Endo F. Markers associated with inborn errors of metabolism of branched-chain amino acids and their relevance to upper levels of intake in healthy people: an implication from clinical and molecular investigations on maple syrup urine disease. J Nutr. 2005;135:1565–70.

12. Hamosh A, Scott AF, Amberger J, Valle D, McKusick VA. Online Mendelian Inheritance in Man (OMIM). Hum Mutat. 2000;

13. Den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: A discussion. Hum Mutat. 2000;

14. Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN, et al. Automated inference of molecular mechanisms of disease from amino acid substitutions. Bioinformatics. 2009;25:2744–50.

15. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. Genome Res. 2001;

16. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods. 2014;

17. Schadewaldt P, Bodner-Leidecker A, Hammen HW, Wendel U. Significance of L-alloisoleucine in plasma for diagnosis of maple syrup urine disease. Clin Chem. 1999;45(10):1734–40.

18. Herber S, Schwartz IVD, Nalin T, Oliveira Netto CB, Camelo Junior JS, Santos ML, et al. Maple syrup urine disease in Brazil: A panorama of the last two decades. J Pediatr (Rio J) [Internet]. 2015;91(3):292–8. Available from: http://dx.doi.org/10.1016/j.jped.2014.08.010

19. Hoffmann G, Schulze A. Organic Acidurias. Pediatric Endocrinology and Inborn Errors of Metabolism. 2009.

20. Morton DH, Strauss KA, Robinson DL, Puffenberger EG, Kelley RI. Diagnosis and Treatment of Maple Syrup Disease: A Study of 36 Patients. Pediatrics. 2002;

21. Wilson JMG, Jungner G. Principles and Practice of Screening for Disease [Internet]. world health organization. 1968. Available from: http://apps.who.int/iris/bitstream/handle/10665/37650/WHO_PHP_34.pdf?sequence=17&isAllowed=y

22. Yoshino M, Aoki K, Akeda H, Hashimoto K, Ikeda T, Inoue F, et al. Management of acute metabolic decompensation in maple syrup urine disease: a multi-center study. Pediatr Int. 1999;

23. Díaz VM, Camarena C, De La Vega Á, Martínez-Pardo M, Díaz C, López M, et al. Liver transplantation for classical maple syrup urine disease: Long-term follow-up. J Pediatr Gastroenterol Nutr. 2014;

24. Feier FHH, Miura IKK, Fonseca EAA, Porta G, Pugliese R, Porta A, et al. Successful domino liver transplantation in maple syrup urine disease using a related living donor. Brazilian J Med Biol Res. 2014;47(6):522–6.

25. You Y, Sun Y, Li X, Li Y, Wei X, Chen F, et al. Integration of targeted sequencing and NIPT into clinical practice in a Chinese family with maple syrup urine disease. Genet Med [Internet]. 2014;16(8):594–600. Available from: http://www.nature.com/doifinder/10.1038/gim.2013.197%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/24603436

26. Li X, Ding Y, Liu Y, Ma Y, Song J, Wang Q, et al. Eleven novel mutations of the BCKDHA, BCKDHB and DBT genes associated with maple syrup urine disease in the Chinese population: Report on eight cases. Eur J Med Genet [Internet]. 2015;58(11):617–23. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1769721215300288
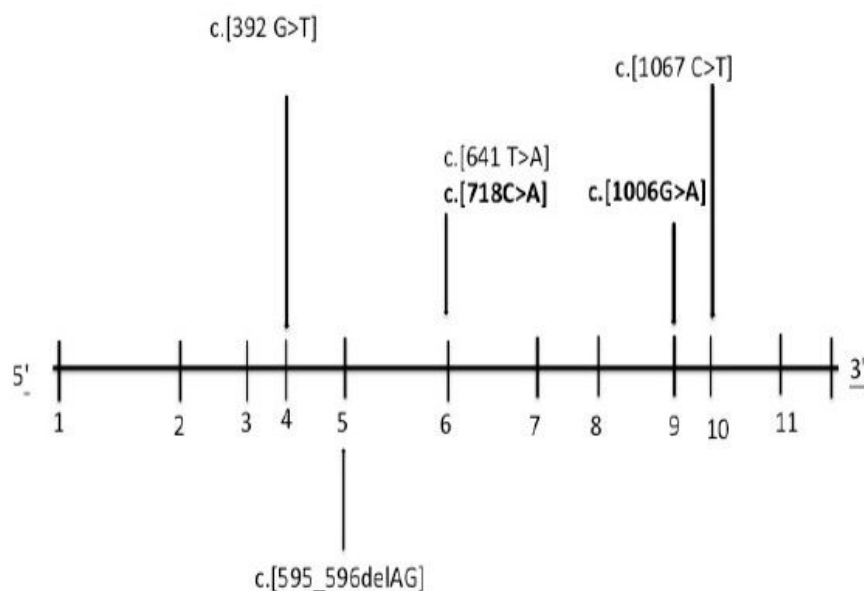
Figures

Figure 1

Schematic representation of BCKDHB and mutations; the new mutations are presented in bold. black lines with numbers: coding exons; black horizontal lines: introns; untranslated regions (5' and 3' UTR)
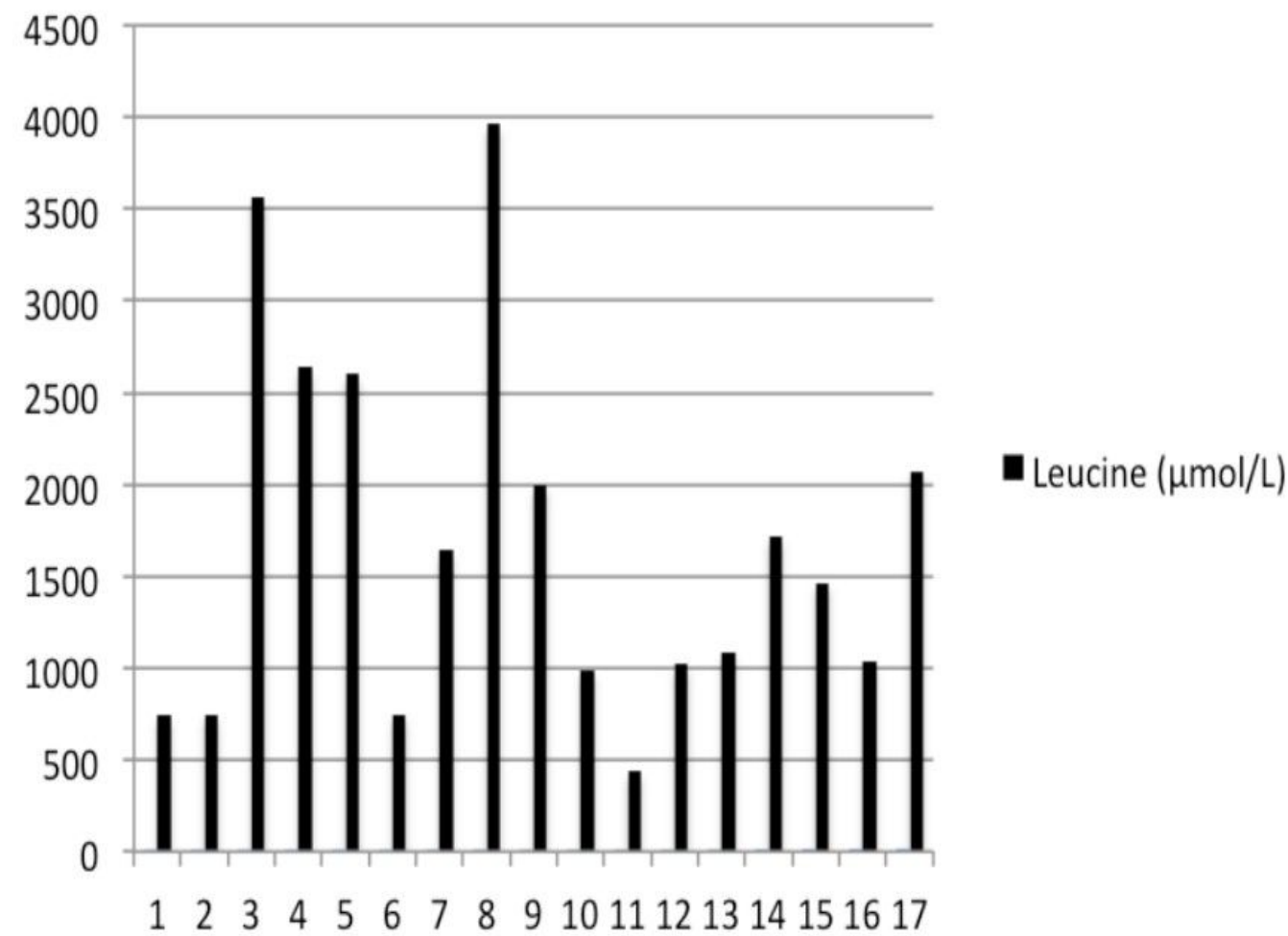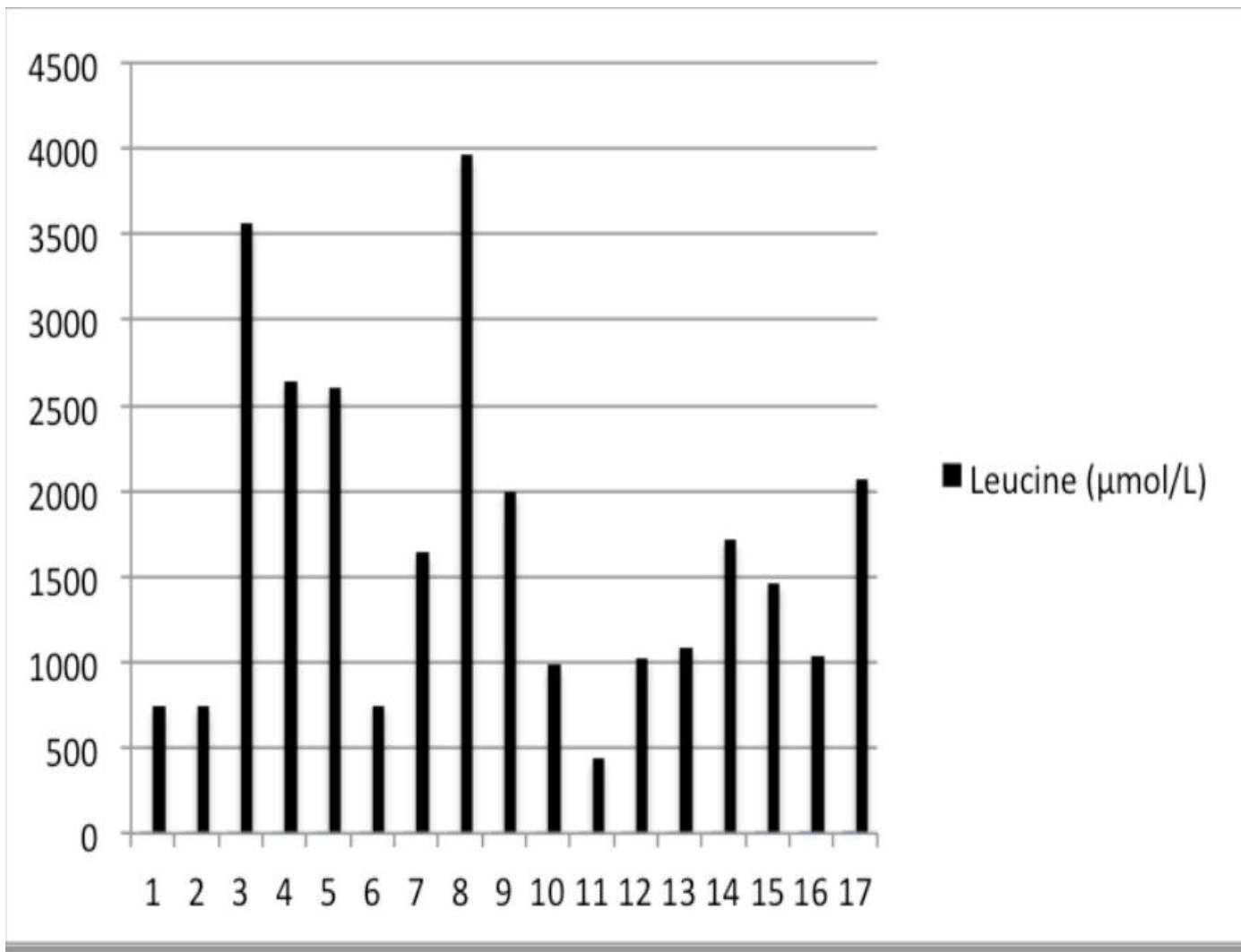

Figure 2

Not available

Figure 3

Representation of leucine values in μmol/L at diagnosis in patients classified with the classic phenotype, according to age at diagnosis
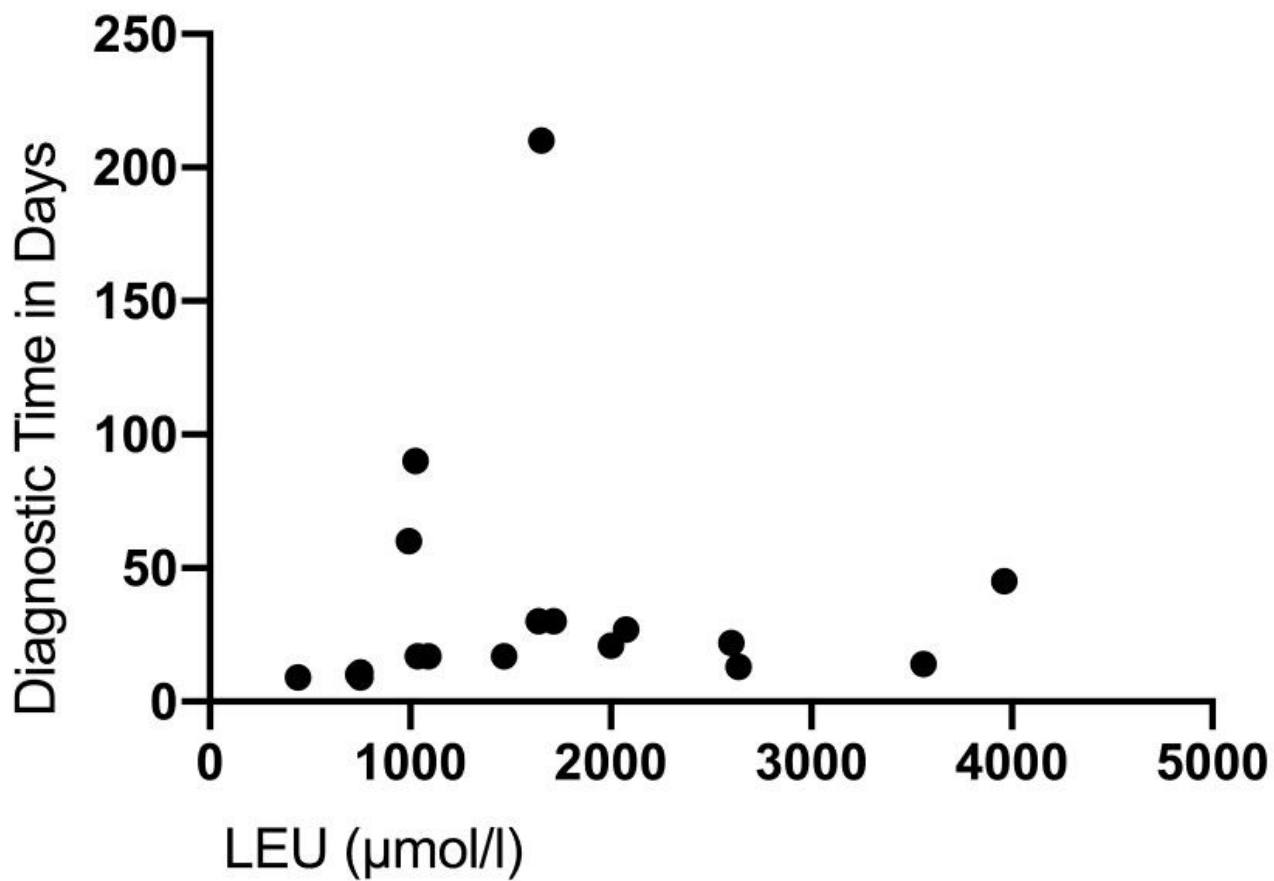
Initial Leucine Values X Diagnostic Time

Figure 4

Correlation between leucine values in µmol/L and age at diagnosis in days, with a p value of 0.989.