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          | Mutation Pathogenicity |
|---------|--------|---------------------------------------------|----------------------------|------------------------|
| 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 | [641 T>A] + [1006 G>A]                      | p. Ile214lys + Gly336S      |                         |
| 4       | BCKDHB | c.595_596delAG + [641 T>A]                  | p. Pro200Stop + Ile214Lys   |                         |
| 5       | BCKDHB | [641 T>A] + [641 T>A]                       | p. Ile214lys + Ile214Lys    |                         |
| 6       | DBT    | 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 | [641 T>A] + [641 T>A]                       | p. Ile214lys + Ile214Lys    |                         |
| 10      | BCKDHB | c.[392 G>T] + [595_596delAG]                | p. Gly131Val + Pro200Stop   |                         |
| 11      | BCKDHB | [641 T>A] + [641 T>A]                       | p. Ile214lys + Ile214Lys    |                         |
| 12      | BCKDHB | [641 T>A] + [641 T>A]                       | p. Gly131Val + Gly131Val    |                         |
| 13      | BCKDHB | [392 G>T] + [392 G>T]                      | p. Pro200Stop + Ile214Lys   |                         |
| 14      | BCKDHB | [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 | [641 T>A] + [641 T>A]                       | p. Ile214lys + 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.

| Exon | Gene   | Nucleotide                                  | Protein Prediction | Type | Origin | Phenotype | Reference                   |
|------|--------|---------------------------------------------|--------------------|------|--------|-----------|-----------------------------|
| 4    | BCKDHB | c.[392 G>T]                                 | p.Gly131Val        | M    | NA     | Classic   | Margutti AV, 2015           |
| 5    | BCKDHB | c.[595_596delAG]                            | p.Pro200Stop       | D    | Spanish| Classic   | Henneneke M, 2003           |
| 6    | BCKDHB | [641 T>A]                                   | p.Ile214lys        | M    | Spanish| Classic   | Rodriguez-Pombo,2006        |
| 10   | BCKDHB | [1067 C>T]                                  | p.Pro356Leu        | M    | Portuguese| Classic   | Quental, 2008               |

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 |
|--------|--------|-------------|--------------------|------|---------------|-----------------|-------------------------|
| Exon 8 | BCKDHA | c.[1013C>T] | p.Thr338Ile        | M    | Pathogenic    | Pathogenic      | 1/18                    |
| Exon 6 | BCKDHB | c.[718C>A]  | p.Pro240Thr        | M    | Pathogenic    | Pathogenic      | 3/18                    |
| Exon 9 | BCKDHB | c.[1006G>A] | p.Gly336Ser        | M    | Pathogenic    | Pathogenic      | 1/18                    |
| Exon 10| DBT    | c.[1217G>A] | p.Gly406Asp        | M    | Pathogenic    | Pathogenic      | 1/18                    |

M: Missense.

Clinical Analysis

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                    | Hepatic Cholestasis     |
| 8       | 2830             | 49                | AGA                    | no                      |
| 9       | 3150             | 50                | AGA                    | no                      |
| 10      | 2600             | 47                | AGA                    | 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.
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.

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/Sev       | 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      | Phenotype   | Total | P-Value |
|------------------|------------|-------------|-------|---------|
| Others           |            | Light/Moderate | 1     | 6       | 7      | 0.282 |
| Ile214Lys        |            | Severe/Death   | 4     | 4       | 8      | 0.050 |
| Mutation          |            | Intermediate  | 1     | 6       | 7      | 0.245 |
| Others           | Phenotype  |              |       |         |       |       |
| Ile214Lys        |            | Classic       | 0     | 11      | 11     | 0.245 |
| Allele           | NPMDD      |              |       |         |       |       |
| Heterozygous     | Light/Moderate |            | 3     | 2       | 5      | 0.394 |
| Homozygous       | Severe/Death |              | 1     | 2       | 3      |       |
| Allele           | Phenotype  |              |       |         |       |       |
| Heterozygous     | Intermediate |            | 0     | 6       | 6      | 0.245 |
| Homozygous       | Classic    |              | 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.Thr338lle, 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
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 student who performed the participants biomolecular study and analysed the
correlations 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 responsible for collecting 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 interpretation for the genotype/phenotype correlation. All authors read and approved the final manuscript.

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