Molecular basis of various forms of maple syrup urine disease in Chilean patients

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Funding information
This research was funded by the Brazilian MSUD Assistance and Research Network. They provided the financial resources that facilitated the data collection.

ABSTRACT
Background: Maple syrup urine disease (MSUD) is an autosomal recessive inherited metabolic disorder caused by the 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 which encode the E1α, E1β, and E2 subunits of the BCKD complex, respectively. The aim of this study was to characterize the genetic basis of MSUD in a cohort of Chilean MSUD patients by identifying point mutations in the BCKDHA, BCKDHB, and DBT genes and to describe their impact on the phenotypic heterogeneity of these patients.

Methods: This manuscript describes a cross-sectional study of 18 MSUD patients carried out using PCR and DNA sequencing.

Results: Four novel pathogenic mutations were identified: one in BCKDHA (p.Thr338Ile), two in BCKDHB (p.Gly336Ser e p.Pro240Thr), and one in DBT

Abbreviations: BCAA, branched-chain amino acids; BCKA, branched-chain keto acids; 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; CNS, central nervous system; DBT, dihydrolipoamide branched chain transacylase E2; DLD, dihydrolipoamide dehydrogenase; DNA, deoxyribonucleic acid; IEM, inborn error of metabolism; ILE, isoleucine; INTA, Nutrition and Food Technology Institute, Dr. Fernando Monckenberg Barros, Chile University; LEU, leucine; MSUD, maple syrup urine disease; NPMDD, neuropsychomotor developmental delay; PCR, polymerase chain reaction; VAL, valine.

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Maple syrup urine disease (MSUD) (OMIM #24860) is an inborn error of the metabolism (IEM) caused by a deficiency in the activity of the branched-chain keto acid dehydrogenase (BCKD) complex which results in the 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) (Strauss et al., 2006). 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 (Chuang et al., 2004). The accumulation of these amino acids mainly affects the central nervous system (CNS) (Chuang et al., 2004). There are already a number of described mutations that have been linked in to MSUD most of these involve disturbance in the catalytic subunits of the branched chain α-keto acid dehydrogenase (BCKD) complex. Based on the analysis of the altered loci, MSUD can be divided into three genetic subtypes: type Ia (MIM # 608348) for mutations in the BCKDHA gene (subunit E1α), type Ib (MIM #248.611) mutations found in the BCKDHB gene (subunit E1β), and type II (MIM # 248610), with mutations in the DBT gene (subunit E2) (McKusick, 2007; Rodríguez-Pombo et al., 2006). BCKDHA has been mapped to human chromosome 19, at 19q13.2. This gene encompasses approximately 27.2 kb of DNA, with a coding sequence distributed across 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 is found at 1p31, comprises 63 kb of DNA, also has 11 exons and a coding sequence of 10,831 bp (Chuang et al., 2001; Stenson et al., 2014). Between these three genes, researchers have identified more than 140 mutations in the literature (Quental et al., 2010). MSUD is an autosomal recessive disorder with a global incidence rate of approximately one in 185,000 newborns. Although it is a rare defect, in some Mennonite populations settled in Pennsylvania, and a handful of other cities in the United States, the estimated elevated incidence is one in 200 live births (Chuang et al., 2001). A study carried out in Portugal by Quental et al. (2010), which evaluated cases diagnosed by mass spectrometry, and found an incidence of one per 86,800 newborns (Margutti, 2015). 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) (Mitsubuchi et al., 2005).

The clinical manifestations of patients with MSUD are varied and depend on the levels of residual enzyme activity. The clinical phenotypes associated with MSUD are classified as classic, intermediate, intermittent, responsive to thiamine therapy, and lipoamide dehydrogenase deficiency (E3 subunit), depending on, among other criteria, the age of onset and severity of the disease. In the classical form of MSUD, patients present with less than 3% residual enzyme activity and symptoms appear soon after birth. Patients typically have ketosis and LEU plasma concentrations of more than 2000 μmol/L. In untreated newborns, maple syrup odor can be detected in the earwax within the first 12–24 hr, and in urine 48–72 hr after birth, although this characteristic odor is variable and thus not a reliable diagnostic aide. Elevated plasma concentrations of BCAA, as well as widespread disturbances in the plasma concentrations of amino acids are present at 12–24 hr of age; elevation of keto acids and ketonuria and irritability can be observed 24–72 hr after delivery; encephalopathy manifesting as lethargy and intermittent breathing difficulties are seen after 4 to 5 days; and coma and central respiratory failure can occur between 7 and 10 days (Strauss et al., 2006).

The intermediate form of MSUD features in infancy and childhood, is characterized by psychomotor developmental delay, failure to thrive, seizures, and walking difficulty. Ketosis and plasma concentrations of LEU less than 2000 μmol/L are typical. Although BCAA elevation is persistent, and there is neurological impairment, severe newborn organ decompensation is not seen like in the classical form.
Enzymatic activity in these cases is between 3% and 30% (Chuang et al., 2004).

Clinically, intermittent MSUD usually presents at around 5 months to 2 years of age, with symptoms following an intercurrent febrile illness. Neurological manifestations during such metabolic derailment may include ataxia, drowsiness, lethargy and/or coma, and the crises may be fatal if untreated (Pode-Shakked et al., 2020).

The sensitivity to thiamine form has a clinical presentation similar to intermediate and intermittent cases, without acute decompensation. Thiamine is a subunit E1 cofactor, regulating the activity of the enzyme complex. Thus, the administration of thiamine decreases serum levels of BCAA. The doses of thiamine used may vary from 10 to 1000 mg per day (Hamosh et al., 2002).

The E3 subunit deficiency form of MSUD is very rare, having been reported in approximately 20 cases from around the world. The prognosis for this form of MSUD seems to be dependent on the residual enzymatic activity which can be between 0% and 25% (Hamosh et al., 2002).

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

2 | OBJECTIVES

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

3 | MATERIALS AND METHODS

3.1 | Patients

The nutrition team from the Genetics and Metabolic Diseases Laboratory of the Nutrition and Food Technology Institute, Chile University (INTA), established an agreement with Porto Alegre Clinical Hospital, Brazil, in order to perform a collaborative study designed to identify the novel genetic mutations present in a Chilean cohort of MSUD patients.

A total of 36 patients were recruited to the study from June to August 2012. Their DNA was extracted from 5 to 10 ml blood samples and used for genetic analysis.

Porto Alegre Clinical Hospital used the Brazilian MSUD Assistance and Research Network to send these samples to Ribeirão Preto Medical School at São Paulo University in order to have the three main genes involved in MSUD analyzed at a molecular level. To date, DNA from 18 patients has been analyzed.

3.2 | Molecular analysis

DNA was extracted from peripheral blood mononuclear cells for the molecular analysis of the three genes involved in MSUD, \textit{BCKDHA} (NM_000709.3), \textit{BCKDHB} (NM_000056.3), and \textit{DBT} (NM_001918.2), access number from GenBank®, http://www.ncbi.nlm.gov/genbank/.

3.3 | DNA sequencing

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

3.4 | 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 (Den et al., 2000). The nomenclature for sequence variant descriptions were derived using the Human Genome Variation Society guidelines (http://www.hgvs.org/mutnomen) (Li et al., 2009).

To verify the pathogenic potential of the missense mutations, \textit{in silico} analysis was performed using MutPred® v1.2 (Ng & Henikoff, 2001), Polyphen-2®-Polymorphism Phenotyping v2 software, and SIFT® (Schwarz et al., 2014). Sequence variants were also evaluated for their disease-causing potential using the Mutation Taster application (Lizcano Fernández, 2005).

3.5 | Clinical data

The nutrition team from INTA, Chile University, provided clinical data regarding the patients in this study. This data included anthropometry at birth, complications during pregnancy, birth date, age of diagnosis, hospitalization, clinical and laboratory test results for leucine, valine, and isoleucine and social information including ancestry and economic class. All these parameters were used to discuss some of the findings.

3.6 | 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
variation, and to check for the possibility of establishing genotype/phenotype relationships with p-values <0.05.

4 | RESULTS

4.1 | Molecular analysis

Of the 18 patients studied 88% presented with mutations in the BCKDHB gene, one patient had a mutation in the BCKDHA gene, and one patient harbored a mutation in DBT. A total of eight mutations were found in the samples, and four of these (50%) were novel. The novel mutations were p.Gly336S and p.Pro240Thr, in BCKDHB, p.Thr338Ile in BCKDHA, and p.Gly406Asp in DBT (Table 1).

This study was able to identify the highest incidence of mutations in exon 6 of BCDKB.

4.2 | Mutation pathogenicity

Among the mutations already described in the literature, the p.Ile214Lys mutation, of Spanish origin, had the highest incidence, totaling 61% of the patients, followed by mutation p.Pro200Stop, also of Spanish origin, in 33% (Table 2). Mutations p.Gly131Val, p.Pro200Stop, and p.Ile214Lys were found to be heterozygous, while p.Gly131Val, and p.Ile214Lys were found to be homozygous.

Novel mutation p.Pro240Thr was found in 16% of the samples; and was located in exon 6 of BCKDHB. This was the most prevalent of the novel mutations. Following in silico analysis all the novel mutations were classified as pathogenic (Table 3). Mutations p.Thr338Ile, p.Gly406Asp and p.Pro240Thr were detected in heterozygous patients while p.Pro240Thr and p.Gly336Ser were found in homozygous patients.

4.3 | Clinical analysis

4.3.1 | Anthropometric assessment

According to clinical data, 72% of the children in this study were born with weight and lengths appropriate for their gestational ages, based on the Intergrowth 21st scale. Patient six was born weighing 4 kg, which is characterized as macrosomia, common in newborns of pregnant women who have gestational diabetes, which was the case here. Patient ten was born with insufficient weight, but her mother had no problems during pregnancy. The mother of child number 14 presented with preeclampsia and this patient had a low birth weight.

| Patient | Gene | Nucleotide | Protein Prediction |
|---------|------|------------|-------------------|
| 1       | BCKDHA | c.[1013 C > T]^a +[1013 C > T]^a | p.Thr338Ile + Thr338Ile |
| 2       | BCKDHB | c.[595_596delAG] + [641 T > A] | p.Pro200Stop +Ile214Lys |
| 3       | BCKDHB | c.[641 T > A] + [1006 G > A]^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]^a + c.[1217 G > A]^a | p.Gly406Asp+Gly406Asp |
| 7       | BCKDHB | c.[595_596delAG] + [641 T > A] | p.Pro200Stop + Ile214Lys |
| 8       | BCKDHB | c.[718 C > A]^a + [718 C > A]^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]^a + [718 C > A]^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 |

BCKDHA (NM_000709.3).
BCKDHB (NM_000056.3).
DBT (NM_001918.2).

^aNew Mutations.

TABLE 1 Pathogenic variants detected in BCKDHA, BCKDHB, and DBT genes of Chilean MSUD patients
4.3.2 | Clinical evaluation

According to the symptom’s onset age, 95% of the patients in this cohort were considered to have the classic form of the disease, and 5% the 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, with four presenting with mild NPMDD, three with moderate NPMDD and six with severe NPMDD. Only two children did not have NPMDD, and that information was not available for three participants. Therefore, in children for whom NPMDD data was available, 40% had a serious degree of delayed neuropsychomotor development.

The biochemical test values at the time of diagnosis provided by INTA were all relatively high. Leucine levels ranged from 440 to 3962 μmol/L at diagnosis (normal range 35–270 μmol/L). Neuropsychomotor Developmental Delay (NPMDD) occurred in 13 of the 18 children studied, with four presenting with mild NPMDD, three with moderate NPMDD and six with severe NPMDD. Only two children did not have NPMDD, and that information was not available for three participants. Therefore, in children for whom NPMDD data was available, 40% had a serious degree of delayed neuropsychomotor development.

The most prevalent signs and symptoms in this cohort included axial hypotonia, in 83% of the children, followed by NPMDD in 77% of the cases, intellectual deficit in 61%, 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, mucous, and skin lesions in 16%, attention deficit disorder and hyperactivity in 14%, extrapyramidal syndrome in 11%, and coma in 5%.

4.4 | 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. One patient was classified with an intermediate form of MSUD, with diagnosis at 90 days of age, and 17 patients were assigned to the classical form, with diagnosis age ranging from 9 to 30 days (Table 5). The clinical presentation of these patients varied, exhibiting NPMDD and poor prognosis, leading in some cases to death. Among the patients with the classical MSUD phenotype, four died, six had severe NPMDD, two had moderate NPMDD, and two were diagnosed with mild NPMDD. In the intermediate patient NPMDD was mild.

Leucine level values were highly variable. Even in patients with classical presentation these values ranged from 741 to 3962 μmol/L, as shown in Figure 1 and Table 6.

In the case of homozygous mutations for c. [1013 C > T] or p.Thr338Ile (novel), c. [1217 G > A] or p.Gly406Asp (novel), c. [392 G > T] p.Gly131Val, c. [1006 G > A] or p.Gly336Ser, and c. [1217 G > A] or p.Gly406Asp (novel), c. [1067 C > T] or p.Pro356Leu, and c. [641 T > A] or p.Ile214Lys 100% of cases presented in as classical MSUD. Homozygous c. [718 C > A] or p.Pro240Thr mutations were associated with intermediate MSUD in 50% of cases. All heterozygous mutations were found in patients with classic phenotypes.

In addition, we attempted to draw a correlation between leucine values and diagnostic age, but this was not possible as a result of the high degree of variability in these values (Figure 2). Mutation p.Ile214Lys was identified in 11 of the 18 samples, which meant that it was possible to combine clinical
information regarding these patients in an attempt to establish genotype/phenotype correlations. The 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.
phenotypes were assessed using Fisher’s exact test. None of these values correlated with a probability value of less than the 5% cutoff value. Thus we were not able to draw any conclusions around genotype/phenotype correlations (Table 7).

5 | DISCUSSION

Only 5% of the patients in this study presented with an intermediate form of MSUD, while the majority, 95%, had the classic form of the disease. These classifications were made primarily as a result of the early onset of symptoms, with a high prevalence of homozygous mutations, 61%. It is interesting that none of the patients had consanguinity involving the parents, although in two cases there were historical reports of illness in the family. This fact might be linked to the greater genetic homogeneity in the Chilean population originating from their indigenous origin. The overwhelming majority of Chileans are the product of varying degrees of admixture between European ethnic groups (predominantly Spaniards) with Amerindian, people indigenous to Chile’s territory.
Although the historical mixing of Europeans and Amerindians is evident across all social strata in the Chilean population, there is a strong correlation between the ratio of a Chilean’s European and Amerindian genetic components and his or her socioeconomic situation (Lizcano Fernández, 2005).

There is a marked increase in Amerindian ancestry in the lower social classes in Chile, with an increasing European component in the upper classes. Indigenous inheritance, whether cultural or genetic, is most pronounced in rural areas and in aspects of the culture including Chilean cuisine and the Chilean Spanish language (Lizcano Fernández, 2005).

The largest portion of the participants in this study were from Santiago (44%), Chile’s capital. With 73% considered lower class and 27% middle class. Their ancestry was predominantly Spanish (88% of cases). Although none of them had a surname of indigenous origin, it is known that the vast majority of this population is a genetic admix.

An autosomal DNA study from 2014 found that Chile possesses a gene pool with an average admix of 51.85% (± 5.44%) European, 44.34% (± 3.9%) Amerindian, and 3.81% (± 0.45%) African. This study was conducted across all regions of Chile. When this data was stratified by social class and region they were able to show that the average Santiago residents admix was strongly influenced by their social class. With the lower class exhibiting an admix of 51% European and 49% Amerindian, the middle class 70% European and 30% Amerindian and the upper class 91% European and 9% Amerindian (Cruz-Coke & Moreno, 1994).

MSUD is not routinely tested as part of a newborn screening program in Chile. It is initially identified by clinical examinations and is often first suspected following detection of the peculiar maple syrup odor in patient’s urine. BCAA serum analysis is the most convenient method for diagnosis and is primarily done during newborn screening. If a patient has high levels of leucine, valine, isoleucine, or alloisoleucine (>5 mm/L), MSUD must be part of a clinicians differential

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

**TABLE 7** Associations between patients with the p.Ile214Lys mutation and specific clinical parameters

| Mutation       | NPMDD       | Light/Moderate | Severe/Death | Total | p-value |
|----------------|-------------|----------------|--------------|-------|---------|
| Others         |             | 1              | 6            | 7     | 0.282   |
| Ile214Lys      |             | 4              | 4            | 8     |         |

| Mutation       | Phenotype   | Intermediate | Classic | Total | p-value |
|----------------|-------------|--------------|---------|-------|---------|
| Others         |             | 1            | 6       | 7     | 0.245   |
| Ile214Lys      |             | 0            | 11      | 11    |         |

| Allele         | NPMDD       | Light/Moderate | Severe/Death | Total | p-value |
|----------------|-------------|----------------|--------------|-------|---------|
| Heterozygous   |             | 3              | 2            | 5     | 0.394   |
| Homozygous     |             | 1              | 2            | 3     |         |

| Allele         | Phenotype   | Intermediate | Classic | Total | p-value |
|----------------|-------------|--------------|---------|-------|---------|
| Heterozygous   |             | 0            | 6       | 6     | 0.245   |
| Homozygous     |             | 0            | 5       | 5     |         |

*Note: p-value < 0.05.*

Others: mutations identified in this study other than p.Ile214Lys.

Abbreviations: NPMDD, neuropsychomotor developmental delay.
diagnosis (Schadewaldt et al., 1999). In addition, levels of leucine greater than 1000 µmol/L are considered critical, as they can result in long term organ damage, or even lead to death (Schadewaldt et al., 1999). Leucine levels within our study ranged from 440 to 3962 µmol/L at the time of diagnosis, and most of the participants (72%) presented with leucine values of more than 1000 µmol/L.

The BCKDHB gene harbored the majority of the mutations, with six of the eight mutations from this cohort located in the E1b subunit. The Spanish mutation p.Ile214Lys was the known mutation with the highest incidence and was detected in 61% of the patients. The novel mutation with the highest incidence was p.Pro240Thr which was identified in 16% of the samples. Notably both mutations occur in exon 6 of BCKDHB.

According to Rodríguez-Pombo and collaborators (2006), p.Ile214Lys mutation is responsible for reducing the stability of the coding protein causing a classic form of the disease. In our findings, we observed symptom variability in patients with this mutation: patients 5, 9, 11, 12, and 18 had a homozygous p.Ile214Lys mutation, while children 9 and 11 presented with serious NPMDD, child five exhibited only mild NPMDD.

Other factors that need to be considered include the level of leucine and the evolution of the disease. It was observed that patient five, with a leucine value of 2600 µmol/L at diagnosis, showed only mild NPMDD, while patient 11, with a leucine level of 440 µmol/L had serious NPMDD. Therefore, we found no association between the initial leucine levels and disease severity. Patients 11 and 12 are siblings, have homozygous p.Ile214Lys mutations and their initial values of leucine were very different. Patient 12 is the eldest, born in 1997; and it took 90 days to establish his MSUD diagnosis, while his younger brother, born in 2006, obtained a diagnosis in only 9 days. This rapid diagnosis was probably aided by the history of illness in the family, improvements in diagnostic testing for MSUD, as well as the previous genetic counseling that this family had already received. The earlier diagnosis almost certainly helped reduce the severity of MSUD.

This kind of symptom variability was also observed in the p.Pro240Thr variant. When this appeared as a homozygous mutation it manifested as serious NPMDD in patient 8 but light NPMDD in patient 15.

High levels of leucine are seen at diagnosis in most critical patients, and this can result in permanent organ damage or even death (Hoffmann & Schulze, 2009). The results of a Brazilian study (20) on the spectrum of MSUD over the last two decades found no significant association between the severity of mental developmental delays and the 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 is a more important factor in cognitive and psychomotor development than initial levels of leucine (Hoffmann & Schulze, 2009).

When evaluating the novel mutations p.Thr338Ile, p.Gly336Ser, and p.Gly406Asp and the age of diagnosis in the participants, most of these mutations seem to result in classic MSUD, with early onset of symptoms. Only p.Pro240Thr was identified in patients (Strauss et al., 2006) with later disease onset, favoring an intermediate phenotype classification. However, one of the major problems observed here is the time between onset of symptoms and the diagnosis in locations without newborn screening for MSUD, like in Chile. One of the patients with a homozygous p.Pro240Thr mutation was classified as an intermediate phenotype because of the diagnosis age but this patient remained in hospital for 90 days. His diagnosis was at 210 days, but how much time passed between onset of symptoms and diagnosis is unknown. It is worth noting, as discussed before, that the BCAA values and clinical evolution of this patient was not consistent with that described in the literature for intermediate phenotypes. Thus, a patient's clinical situation is not necessarily the best way to correlate phenotype and genotype, because neurological deterioration is directly associated with the absence of early diagnosis and, therefore, with the absence of adequate nutritional treatment, which is fundamental to the control of BCAA concentration. In a study by Morton et al. (2002), in which patients had prompt access to a metabolic formula, and where the clinical protocol was followed in the acute early stages of the disease, patient outcomes were better and patients were able to reach more age appropriate developmental milestones (Morton et al., 2002).

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.

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

MSUD treatment includes the application of low protein diets, supplementation with BCAA-free formula and symptomatic treatment during metabolic crises, including ingestion of mannitol to treat brain edema, injection of insulin to lower blood glucose, and use of N-carbamylglutamate to reduce serum ammonia levels (Yoshino et al., 1999).

As the liver is responsible for 15% of BCKD production, liver transplantation can restore enzyme activity in patients with MSUD (Díaz et al., 2014). Liver transplantation may benefit patients with the classical form of the disease; however, it does not reverse the disease process (Feier et al., 2014).

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 a BCKDHA gene mutation; where the fetus underwent prenatal diagnosis (You et al., 2014). In a study by Li et al. (2015), they were also able to identify a BCKDHB mutation in a prenatal screen (Li et al., 2015). In both cases, treatment was started at birth, and diagnosis was possible as a result of the increased awareness of the caregivers and family.

We found a total of eight mutations in this patient cohort, four of which were novel: p.Thr338Ile, p.Gly336Ser, p.Gly406Asp, and p.Pro240Thr. Six of the eight mutations are located in exon 6 of BCKDHB, and this gene is the one identified most frequently identified as mutated in this population. Mutation p.Ile214Lys is present in 61% of the patients and was first described in patients of Spanish origin.

The novel mutations p.Thr338Ile, p.Gly336Ser, and p.Gly406Asp were all identified in patients with early diagnosis and were classified as classical MSUD mutations based on age at diagnosis. Only p.Pro240Thr mutations presented later, allowing patients to be classified as having an intermediate phenotype. The 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 was diagnosed more promptly, possibly via the application of newborn screening, perhaps it would be possible to establish genotype-phenotype associations more efficiently.

ACKNOWLEDGEMENTS
To the DXB Network professionals who contributed to this article, as well as to the Clinical Hospital Genetic Sector team from Porto Alegre, RS, Brazil; To the Molecular Genetics and Bioinformatics Laboratory members and staff from Ribeirão Preto Clinical Hospital, Brazil who helped with molecular analysis and the members of INTA in Santiago, Chile, who participated in the collection of clinical data from the patients and sent the DNA samples, the authors would like to extend our deepest thanks.

CONFLICT OF INTERESTS
The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS
DRRC was the PhD student who performed the biomolecular assays and analyzed the correlations between phenotype and genotype. AVBM standardized the study methodology and helped with the mutation analysis. WASJ helped with the mutations analysis, DFG helped with the methodology standardization, GAM helped with the biomolecular assays, AAM did the sequencing reaction, IVDS helped with the mutation analysis, VC, VH and GC were responsible for collecting the blood samples and all the participant’s clinical data. FS-L helped with the mutation analysis, ESB helped with the biomolecular assays and JSCM acted as DRRC’s 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.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article. For more information, the datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

ETHICAL COMPLIANCE
This research was approved by the Ribeirão Medical School Research Ethics Committee and by the National Commission of Ethics in Research committee (reference number 2.252.930). All collection and analyses were done in accordance with the Helsinki Declaration of 1975. Parents/guardians signed an Informed Consent form giving permission for participation in the study.

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How to cite this article: Campanholi DR, Margutti AV, Silva WA, et al. Molecular basis of various forms of maple syrup urine disease in Chilean patients. Mol Genet Genomic Med. 2021;9:e1616. https://doi.org/10.1002/mgge.3.1616