Shortcomings of Currently Applied —Dietary— Treatment for Patients with Classical Organic Acidemias and Urea-cycle Disorders

Recommendations to improve outcome

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Shortcomings of Currently Applied —Dietary— Treatment for Patients with Classical Organic Acidemias and Urea-cycle Disorders

Recommendations to improve outcome

Tekortkomingen in de huidige toegepaste —dieet— behandeling van patiënten met een klassieke organische acidemie of een ureumcyclus defect

Aanbevelingen om de uitkomst van patiënten te verbeteren

Proefschrift

ter verkrijging van de graad van doctor aan de
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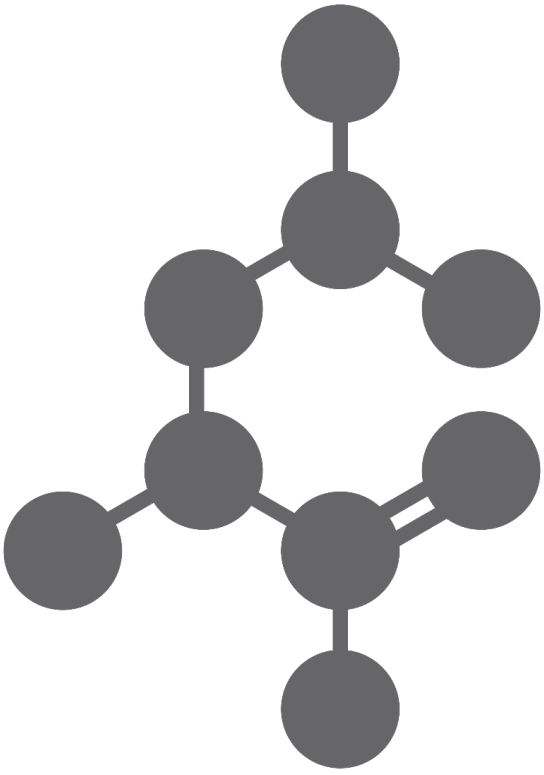


GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

1

Inborn errors of metabolism are rare inheritable disorders with an estimated frequency of 51:100,000 [1]. In the inborn errors of metabolism different groups of metabolic disorders can be distinguished. One of these groups is the intoxication type metabolic disorders. Intoxication type metabolic disorders are characterized by disturbances in glucose metabolism, protein metabolism, or fatty acid metabolism. The research in this thesis will focus on protein metabolism and specifically on the amino and organic acid related disorders (AOA).

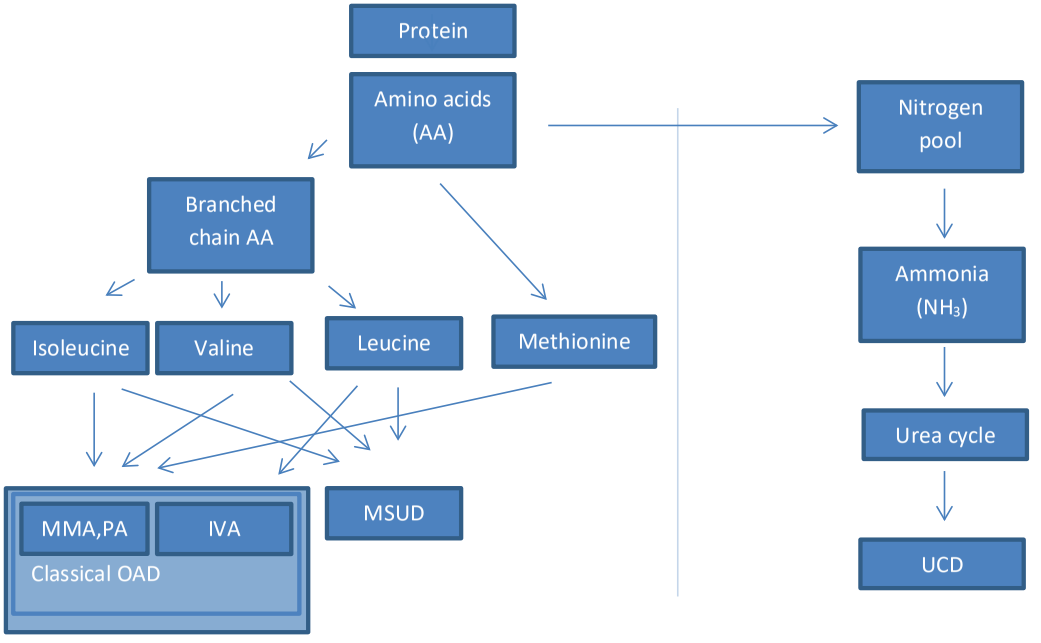
The building blocks of proteins are amino acids, which are essential for human growth and development. Amino acid metabolism results in ammonia production which is processed by the urea cycle and excreted as urea. The AOA compromise the branched chain amino acid (BCAA) disorders and urea-cycle disorders (UCD). The BCAA disorders include classical organic acidemias (OAD) and maple syrup urine disease (MSUD) (figure 1). BCAA disorders are characterized by enzymes deficiencies in specific pathways of the degradation of certain branched chain amino acids. Within the BCAA disorders isovaleric acidemia (IVA) (OMIM#243500), methylmalonic aciduria (MMA) (mutase deficiency OMIM #251000, CblA #251100, CblB #251110), and propionic aciduria (PA) (OMIM #606054) are considered the classical OAD (figure 1). UCD are caused by deficiencies of the enzymes and transporter needed for the urea-cycle (figure 1). While all AOA disorders affect protein metabolism, the disease presentations, diagnostic characteristics and prognosis may differ considerably between and within the subcategories and even between patients with the same disease.

With the currently applied treatment several classical OAD and UCD patients have a poor prognosis. In both disease groups overall mortality risk is high, mainly in those patients presenting in the neonatal period with an acute metabolic decompensation [2]. In classical OAD the majority of patients is symptomatic and a high proportion of the patients develop 1) metabolic decompensations requiring hospital visits, 2) long-term complications, such as cardiomyopathy, renal failure, liver failure, and neurological symptoms, including cerebral palsy and seizures, and 3) intellectual disability [2, 3]. In classical OAD, IVA has been implemented in the newborn screening in 2007 and MMA and PA have been included in the newborn screening in the Netherlands since October 2019. In UCD, numerous patients have recurrent metabolic decompensations, liver failure, failure to thrive, neurological symptoms, including cerebral palsy and seizures, and intellectual disability. Furthermore, psychiatric symptoms such as hyperactivity are observed. In both classical OAD and UCD the quality of

life has been reported to be decreased in some [4, 5], and to be normal in others [6]. Considering the unsatisfactory outcome of classical OAD and UCD patients we need to improve patient care. Since new treatment strategies are still ahead of us, it is essential to evaluate current applied treatment strategies with the aim to improve these in order to ameliorate patient care and outcome.

We hypothesized that with optimizing current available treatment patient outcome can be improved. In order to confirm or decline this hypothesis two main steps were necessary. Firstly, evaluation of current practice needed to be performed (and compared to guideline advices) and secondly it needed to be defined how current practices influences patient outcome. Previous attempts to determine the current practice in classical OAD and UCD patients have been performed. Patient´s height in a Dutch OAD sub-cohort had been evaluated (D. Wensink, unpublished results) and a national cohort study in the Netherlands on UCD patients (n=58) described patient characteristics (D. Salkovic [7]). In the Netherlands, considering the low incidence of these disorders national expertise centers have been installed in some of the academic centers. The Erasmus Medical Center is the center of expertise and approved by the ministry of health for classical OAD and UCD. On an international level, organizations have been set up in Europe specifically addressing AOA, such as the European registry and network for intoxication type metabolic diseases (E-IMD) and the European Reference Network for Hereditary Metabolic disorders (Metab-ERN), which includes a subnetwork for amino acid and organ acids-related disorders (AOA). Regarding OAD and UCD, the E-IMD has set up a registry including 452 classical OAD and 417 UCD patients [8]. Based on the data collected in these registries the disease presentation at disease onset and emerging clinical phenotype has been described [2, 3]. Furthermore, guidelines have been developed, which may help in clinical decision making [9-11]. However, it is not known if guideline advices are followed-up in clinical practice.

FIGURE 1. Amino and organic academia disorders organized. All disorders discussed in this thesis are shown. Classical organic acidemias (OAD) are MMA, PA and IVA. Abbreviations: IVA: isovaleric acidemia, MMA: methylmalonic acidemia, MSUD: maple syrup urine disease, PA: propionic acidemia, UCD: urea-cycle disorders.



1 | Epidemiology of classical OAD and UCD

For OAD the estimated incidence in newborn ranges between 1:48,000-1:61,000 for MMA [12, 13] and between 1:50,000-1:500,000 for PA [13] and has been calculated to be 1:67,000 for IVA[14]. In the Netherlands the incidence of MMA is 1:121,765, of PA 1:188,182 and of IVA 1:180,000 [15]. The estimated incidence of UCD is 1:35,000 births, with a broad variation between the incidence of the different diseases within the UCD subgroups [16, 17].

2 | Pathogenesis and pathophysiology in classical OAD and UCD

In MMA and PA (classical OAD) the degradation of the BCAA isoleucine and valine is affected. PA is caused by propionyl-CoA carboxylase deficiency (encoded by PCAA and PCCB) which is needed for the degradation of propionyl-CoA (formed by degradation of isoleucine and valine) (figure 2). This degradation step performed by propionyl-CoA carboxylase is biotin-dependent (figure 2) [9]. MMA deficiency is either due to a methylmalonyl-CoA mutase deficiency (MCM/Mut) or due to a defect of mitochondrial adenosylcobalamin (CblA and CblB). The MCM types of MMA can be either caused by complete enzyme deficiency (Mut0) or partial enzyme deficiency (Mut-). In these four types of MMA the degradation of l-methylmalonyl-CoA (a product of the degradation of propionyl-CoA) to succinyl-CoA is affected (figure 2). They can be referred to as isolated MMA, and they do cause methylmalonic aciduria with normal homocysteine levels. Other disorders can cause methylmalonic aciduria with hyperhomocystinemia and include the adenosylcobalamine and methylcobalamine defects (CblC and CblD defects) and the lysosomal cobalamin release disorder (CblF) [12]. These last disorders will not be further described in this thesis. Since vitamin B12 (adenosylcobalamin) is a cofactor of MCM, vitamin B12 responsive and unresponsive types of MMA exist (figure 2). It is necessary to notice that the end product (namely succinyl-CoA) is an important substance for the tricyclic acid cycle (figure 3), which is essential for energy production. Besides the degradation of the branched chain amino acids, the degradation of methionine, threonine [18] and carbohydrate and fatty acid oxidation are also affected in MMA and PA [19]. Furthermore the enzymes deficient in these disorders are involved in the catabolism of propionyl-CoA,

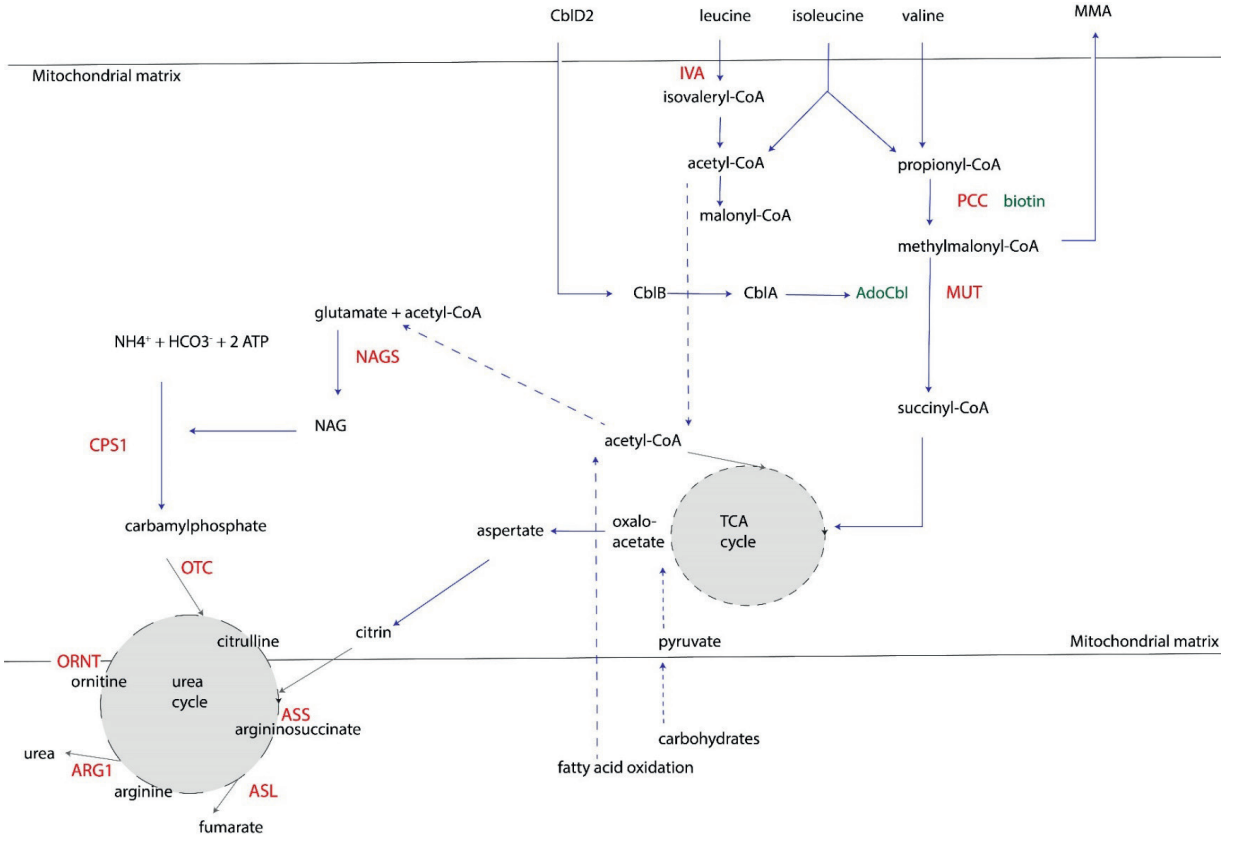
which can be formed in the gut by bacteria and by the degradation of fatty acids and cholesterol side chains. In IVA (classical OAD) the degradation of leucine is affected. The latter disease is caused by a deficiency of isovaleryl-CoA dehydrogenase [20].

In classical OAD, pathophysiology leading to disease manifestation consists of three main principles: 1) the accumulation of toxic metabolites, 2) deficiencies of certain end-products, and 3) mitochondrial dysfunction and oxidative stress. Toxic metabolites are formed due to insufficient degradation of the BCAA and will be discussed further on this thesis. With the degradation of isoleucine and valine normally succinyl-CoA is formed, which is an important product for the TCA cycle and thereby for energy production. The observed mitochondrial dysfunction is likely mainly due to toxicity of metabolites on mitochondria. Furthermore, it is observed that both propionic acid and 2-methylcitric acid can negatively affect the citric acid cycle and respiratory chain complexes.

The urea cycle plays a key role in the detoxification of ammonia, which takes place almost entirely within the hepatocytes [21]. Ammonia detoxification occurs in different steps. First ammonia is converted by carbamylphosphate synthetase 1 (CPS1) to carbamylphosphate. This step needs N-acetylglutamate (NAG), which is produced from glutamate by N-acetylglutamate synthase (NAGS). Carbamylphosphate enters the urea cycle and is then converted to urea, which can be excreted. In the urea cycle, three enzymes are active in the mitochondria, one is active at the mitochondrial membrane and three are active extra-mitochondrial or cytosolic (figure 3). The UCD comprise the inherited deficiency of ornithine transcarbamylase deficiency (OTC-D) (EC 2.1.3.3; OMIM #311250), argininosuccinate synthetase deficiency (ASS-D) (EC 6.3.4.5; OMIM #215700), argininosuccinate lyase deficiency (ASL-D) (EC 4.3.2.1; OMIM #207900), arginase 1 deficiency (OMIM #207800) and hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM #238970). NAGS (OMIM #237310) and CPS1-D (EC 6.3.4.16; OMIM #237300) are also considered to be part of the UCD, however the deficient enzymes are not involved in the urea cycle itself, but rather in the degradation of products that become available for the urea cycle. The urea cycle is regulated mainly by the CSP1 reaction. This reaction is highly dependent on NAG and thereby regulated by glutamate levels. Deficiencies in any of the above mentioned enzymes can cause hyperammonemia. The high ammonia levels that can occur in UCD can cause severe neurological damage. When ammonia

enters the brain it is converted to glutamine by reacting with glutamate. Glutamate can be produced from glutamine and glutamate can also be converted to glutamine. Importantly, the brain cells do not have a urea cycle and glutamine-glutamine cycle is essential for detoxification of ammonia in the brain. Glutamate is an important excitatory neurotransmitter. High glutamate levels can cause hypoxic damage, and even stroke and epilepsy. Due to the buffering of ammonia in UCD, glutamine levels are elevated in UCD and they can cause brain edema by astrocyte swelling [22].

FIGURE 2. Schematic overview of classical OAD and UCD. Abbreviations: ASL: argininosuccinate lyase, ASS: argininosuccinate synthetase, ARG1: arginase deficiency 1, Cbl: cobalamin, CPS1: carbamylphosphate synthetase 1, IVA: isovaleric acidemia, PCC: propionyl-CoA carboxylase, MUT: methylmalonyl-CoA mutase, NAGS: N-acetylglutamate synthetase, OTC: ornithine transcarbamylase, ORNT: ornithine transporter, TCA cycle: Tricarboxylic acid cycle.



3 | Disease presentation in classical OAD and UCD: signs and symptoms

3.1 | First presentation

The majority of classical OAD and UCD patients become symptomatic. They either present with a metabolic decompensation or with long-term complications. In classical OAD 60% of the patients and in UCD 45% of the patients present with a metabolic decompensation in the newborn period [2]. Symptoms of patients presenting in neonatal period are vomiting, dehydration, weight loss, hypotonia or hypertonia, lethargy, seizures and coma [9]. The presentation in the newborn period is often similar to and may be mistaken by a severe neonatal sepsis, which can cause a diagnostic delay. Making a diagnosis in time is essential to optimize patient's outcome. Patients presenting at a later age often present with non-specific symptoms such as feeding problems, mental retardation, epilepsy, movement disorders and for classical OAD also with cardiomyopathy or renal failure [9] and within the UCD spectrum also with psychiatric disorders [2]. MMA and PA patients often have a more severe disease presentation than IVA patients [18].

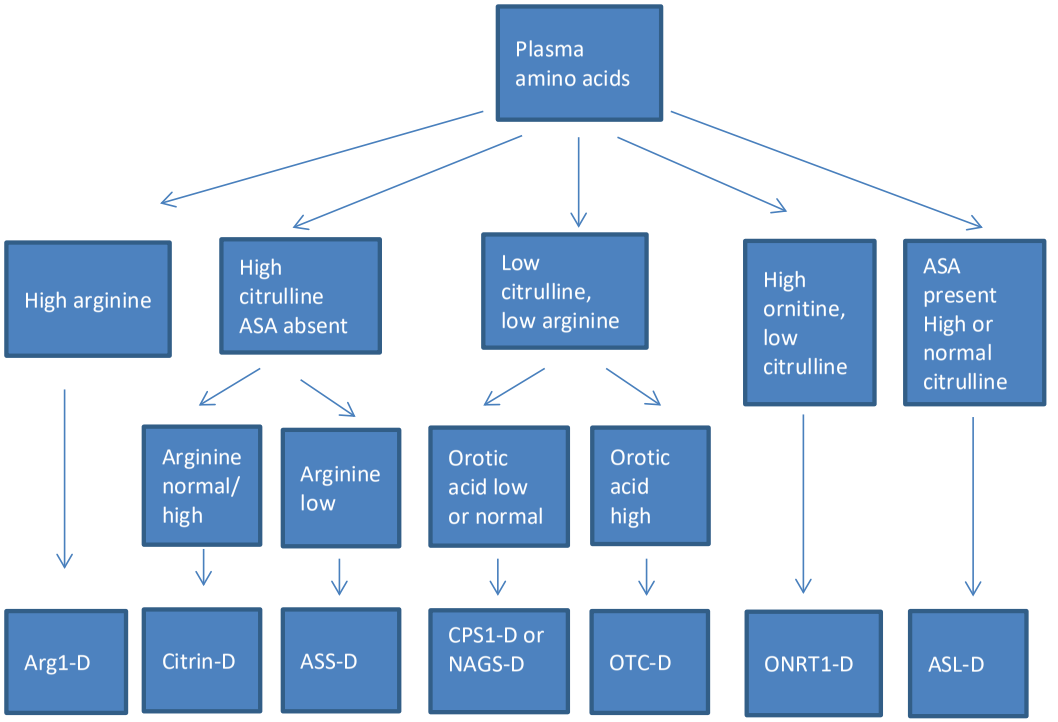
3.2 | The diagnosis

Patients with classical OAD typically present with metabolic acidosis (although this can be absent) with either normal ammonia levels or hyperammonemia (depending on the degree of metabolic decompensation). Hyperammonemia can be due to the fact that propionyl CoA can block the urea cycle by inhibiting N-acetylglutamate synthesis. Furthermore, in classical OAD, elevated anion gap, ketosis, elevated lactate, leukopenia, thrombocytopenia, and anemia can be observed [9]. Diagnostics include determination of plasma amino acid concentrations and plasma or blood spot acylcarnitin levels and urinary organic acid and orotic acid levels. The toxic metabolites in both MMA and PA are propionyl-CoA, propionic acid, hydroxypropionic acid, propionylcarnitine, propionylglycine, and 2-methylcitrate. Disease specific toxic metabolites in MMA are increased methylmalonic acid and methylmalonylcarnitine (which are not increased in PA). Glycine and lysine levels are often elevated and determination of total plasma homocysteine can be helpful in differentiating between different MMA subtypes [9].

In IVA plasma acylcarnitines show an increased C5 and in urine isovalerylglycine is elevated [23]. Enzyme diagnostics and genetic analyses are needed to confirm the diagnosis.

In the neonatal period patients with UCD typically present with hyperammonemia and sometimes metabolic acidosis. Timely diagnosis is essential since hyperammonemia can cause brain damage and even death [21]. Plasma amino acid concentrations (arginine, alanine, citrulline, glutamine) and carnitines, liver enzymes and coagulation factors should be determined as well as urinary organic acid, amino acid and orotic acid levels [24]. The location of the enzymatic defect within the urea cycle determines which amino acid will accumulate, for example arginine accumulates in case of arginase 1 deficiency (Arg1) (figure 2). Figure 3 presents the typical laboratory characteristics in the different UCD (depending on the enzymatic defect within the urea cycle), which helps making the diagnosis of a particular diseases. Hereafter enzyme diagnosis and genetics should be performed.

FIGURE 3. Diagnosing UCD. Schematic overview of abnormalities in plasma amino acid levels in the specific urea-cycle disorders. Abbreviations: ASL-D: argininosuccinate lyase deficiency, ASS-D: argininosuccinate synthetase deficiency, ARG1-D: arginase 1 deficiency, CPS1-D: carbamylphosphate synthetase 1 deficiency, NAGS-D: N-acetylglutamate synthetase deficiency, OTC-D: ornithine transcarbamylase deficiency, ORNT1-D: ornithine 1 transporter deficiency (=hyperonithinemia-hyperammoneamia-homocitrullinuria syndrome).



3.3 | Acute management

For classical OAD and UCD, the goal of treatment in patients presenting in the neonatal period is to stabilize the metabolic derangement/acidosis. In patients with both disorders protein intake should be stopped and high kilocaloric intake needs to be established. In case of hyperammonemia dialysis is necessary when ammonia levels exceed 500umol/L [9, 10, 24]. The acidosis can be corrected with intravenous bicarbonate. However, this should be done with caution. If hyperammonemia is present in OAD patients, a combination of L-carnitine, sodiumbenzoate and phenylbutyrate as well as arginine, carbamylglutamate, vitamin B12 and biotine should be given depending on the severity of the hyperammonemia. It should be noted that the use of sodiumphenylbutyrate or phenylacetate can decrease the levels of glutamine and therefore prescription of these medications should be done after careful consideration [9]. In UCD, nitrogen excretion should be improved by medical treatment with N-scavengers such as nitrogen sodiumbenzoate, sodiumphenylbutyrate/ glycerolphenyl-butyrate, L-arginine or L-citrulline [24]. While sodiumphenylbutyrate can be giving intravenously, glycerolphenylbutyrate can only be administered orally.

3.4 | Metabolic decompensations

In both classical OAD and UCD, metabolic decompensations can occur at any age and they can cause severe morbidity and mortality. The metabolic decompensations can be triggered by catabolic stress due to infections, increased protein intake as well as fasting, surgical procedures and specific medications [9, 24]. However in many patients metabolic decompensations may also occur without a clear trigger and often a combination of more than one trigger is causative [25]. Over-restriction of dietary protein intake can also induce catabolism and thereby trigger a metabolic decompensation [25].

3.5 | Long-term complications

In classical OAD, long-term complications affect the following organ systems: neurological, optical, cardiac, renal, ear, gastro-intestinal (liver, pancreas, gut), skeletal, bone and bone

marrow (joints), fertile (ovaries) and endocrine (growth). Importantly, long-term complications may also occur in metabolic stable patients [3]. Symptoms arising from the neurological system are spasticity/dystonia, movement disorders, epilepsy, hearing or visual field defects and developmental delay – the latter will be discussed in a separate paragraph –. Movement disorders were observed in 7%-27% in OAD patients [3]. Seizures were mainly observed in PA patients (13%) [3]. Metabolic decompensations as well as chronic toxicity can cause brain damage, often observed as basal ganglia hyperintensity on brain imaging, but other abnormalities such as white matter leasions can be found as well [26]. Psychiatric symptoms that can occur are autism, psychosis, and attention deficit hyperactivity disorder (ADHD).

Cardiomyopathy (mainly in PA), renal failure (mainly in MMA)[9], optic nerve atrophy eventually leading to blindness and hepatomegaly are long-term complications likely induced by mitochondrial dysfunction in MMA and PA. Furthermore, it is plausible that prolonged QTc interval (mainly PA), exercise intolerance and muscular hypotonia, epilepsy, pancreatitis (MMA>PA), sensineural hearing loss and bone marrow dysfunction can be caused by mitochondrial dysfunction. Other long-term complications without a clear role of mitochondrial dysfunction are decreased bone mineral density, joint hypermobility, pes planus, growth retardation, skin problems, and enamel defects [26].

In UCD, long-term complications include neurological symptoms, psychiatric symptoms and liver and sometimes renal involvement. Neurological symptoms include movement disorders (such as cerebral palsy), and seizures, as well as developmental delay. Those patients with severe hyperammonemia and neonatal onset have the highest risk of neurological impairment [27]. Movement disorders were observed in 7-25% of the UCD patients [3]. Arg1 and HHH syndrome patients can have diplegia and in Arg1 also tremor, ataxia, choreathetosis can be observed [21]. Seizures were mainly observed in OTC-D male patients (14%) and ASL-D patients (19%) [3]. Psychiatric symptoms such as ADHD, autism, and mood disorders may also be observed. Renal failure is observed in ASL-D and chronic liver disease occurs mainly in OTC-D females and ASL-D [3]. Furthermore, some of the patients may have visual or hearing impairment [28].

3.6 | Cognitive and motor development

In OAD, developmental delay was observed in approximately a quarter (25-65%) of the MMA patients, 59-100% of the PA and 10% IVA patients (identified by selective screening) [9, 29]. Cognitive impairment with an IQ below 70, based on standardized IQ test, was observed in 31% of the OAD patients [6]. MMA vitamin B12 unresponsive patients appeared to have an increased risk of severe impaired cognitive function if they had an early onset hyperammonemia and/or seizures [9]. In PA no clear risk factors could be identified. Abnormal gross motor development was observed in approximately 1/3 of the MMA and PA patients, in only 4% of the IVA patients and fine motor development was abnormal in approximately 40% of the MMA and PA patients.

In UCD, cognitive impairment (IQ<70) was observed in 43% [6] of the patients in the European cohort and intellectual and developmental disabilities in 39% of the cohort described for the USA [28]. [30] Gross motor development was abnormal in 3-50% and fine motor development was abnormal in 9-58% of the patients. The percentage was dependent on the type of disease [3].

3.7 | Quality of life

Classical OAD and UCD patients have shown to be at risk of a decreased health related quality of life [6, 31]. Several factors play a role in the quality of life of OAD and UCD patients and families. The most important ones are caused by the disease itself and are dietary restriction, hospitalizations due to metabolic decompensations, and the occurrence of severe organ related complications that affect long-term outcome.

3.8 | Mortality

While in OAD survival has increased in recent decreased, this is not true for UCD [32]. The survival rate in MMA and PA patients ranges from 70-90% [33-35]. UCD patients presenting in

neonatal period had a survival of only 35%, while those presenting thereafter had a survival of 87% [36].

4 | Long-term treatment in classical OAD and UCD

For both classical OAD and UCD patients, the goal of the dietary management is to reduce the amount of toxic metabolites in order to prevent metabolic decompensations (OAD)/hyperammonemia (UCD) and long-term complications [9, 10]. This can be achieved by reducing the intake of specific amino acids (in classical OAD) and total protein (in classical OAD and UCD). In both disorders a high caloric diet is prescribed to prevent catabolism, since catabolism is associated with endogenous protein breakdown [10] and thereby induces potential toxic metabolites. Besides the dietary treatment some supportive medication is available. Lastly, solid organ transplantation of the kidney and/or liver are increasingly performed.

4.1 | Dietary treatment

4.1.1 | Dietary intake in general population

Appropriate protein incorporation and protein tolerance is essential for normal growth, body weight and physiological, metabolic and psychological functions [24, 37, 38]. Dietary protein intake stimulates growth through the secretion of insulin-like growth factor 1 [39, 40]. To achieve an adequate nitrogen balance, individual metabolic demands, protein quality of the consumed protein, and protein utilization play a role. The metabolic demand of amino acids can be delivered by 1) degradation of proteins by tissues, 2) the novo synthesis of proteins and 3) by protein intake through the diet. The overflow of amino acids can be broken down via other pathways and through oxidation into nitrogen, which can then be excreted [38]. Considering dietary protein intake, the protein energy interaction is important (the protein: energy ratio or P:E ratio). Increased energy intake can improve nitrogen balance by decreasing protein loss [38].

Advice on protein intake as well as on caloric intake are well described in the WHO/FAO/UNU 2007 reports [41] (table 1). For each age category for both females and males advised protein intake in gram/kg/day and kilocalorie in calories/kg/day are given. Advice on protein intake can be based on three main study types. First nitrogen balance studies are based on the principle that the majority of the nitrogen in the body is derives from protein. Hereby nitrogen gain seems to be equal to protein gain. These studies determine the nitrogen intake and output. The second study type concern carbon balance studies and third the indicator amino acid method. In the WHO/FAO/UNU 2007 reports recommendations are based on nitrogen balance studies.

Energy requirements					Protein requirements*	
Age	kJ/kg/day		kcal/kg/day		Age	g/kg/day
	FAO/WHO/UNU 2007		Converted from FAO/WHO/UNU 2007			
Infants (y)	Males	Females	Males	Females	Infants (y)	
0.5	335	340	80.0	81.2	0.1	1.77
					0.2	1.5
					0.25	1.36
					0.5-1	1.31
Children (y)					Children (y)	
2.5	348	334	83.1	79.8	1-10	0.84-0.90
5.0	315	305	75.2	72.8		
10	275	248	65.7	59.2		
15	230	193	54.9	46.1	11-16	0.92-1.14
Adults (y)					Adults (y)	
(Moderate activity, 70 kg)						
18-29	183	159	43.7	38.0	>16	0.84-0.87
30-59	175	148	41.8	35.3		
Adults (y)						
(Moderate activity, 50 kg)						
18-29	212	180	50.6	43.0		
30-59	212	183	50.6	43.7		

Table 1. WHO guidelines for protein and energy intake. *(table from the guideline by Baumgartner et al. [9])*

4.1.2 | Dietary management in classical OAD and UCD

For both classical OAD and UCD, guidelines providing recommendations on long-term treatment are available. In both guidelines the before mentioned recommendations by the WHO are used [9]. Long-term treatment recommendations imply of a restriction of the so called natural protein, which is derived from protein present in natural/normal dietary sources. Furthermore, classical OAD and UCD patients often receive amino acid mixtures (AAM), which

in case of classical OAD contain amino acids without the specific amino acids that cannot be metabolized by the patient which that specific disorder and in case of UCD contain essential amino acids (EAA).

In classical OAD, the guideline notifies that the ultimate treatment goal is to achieve a natural protein intake of 100% of the recommended daily allowances (RDA) (FAO/WHO/UNU (2007)), and that amino acid mixtures (AAM) should be applied when the recommended natural protein intake of 100%RDA is not obtained [9]. In UCD, the guidelines states that 'the FAO/WHO/UNU 2007 can be used as guide' for protein and energy intake. The authors also mention that protein intake below the recommended quantity can still be adequate but that supplementation of EAA may become necessary. Both guidelines states that frequent monitoring of these patients is essential since not only decompensation and long-term complications should be prevented, but also growth and development should be optimized. Vitamin and minerals should be supplied when there are deficiencies induced by the diet.

4.2 | Medical treatment

In OAD, the goal of medical treatment is 1) to stimulate propionyl-CoA elimination, 2) to reduce production of propionyl-CoA in the gut and 3) to increase activity of the deficient enzyme by providing co-enzymes. Propionyl-CoA elimination can be stimulated by providing L-carnitine by conversion to nontoxic propionylcarnitine. L-carnitine can reduce the toxic metabolites by transforming them and by urinary excretion [9]. Propionyl-CoA production can be reduced by providing antibiotics, mainly metronidazole, through reducing the intestinal bacterial flora that produce propionyl-CoA. In MMA, vitamin B12 responsive forms exist and treatment with vitamin B12 clearly improves the prognosis of these patients. In PA it is not yet clear if biotin responsive forms of PA do exist [9]. Besides these treatment options, treatment regarding improving supplementation to the TCA cycle has been studied in PA patients, since in PA (and MMA) the supply of succinyl-COA is deficient. Citrate has shown to improve TCA cycle function in PA patients [42], but is not recommended as standard treatment. In OAD steroids should be avoided since they can induce catabolism. Furthermore, sodium valproate, drugs with propionate, pivalic acid, nephrotoxic drugs, and chemotherapy should be avoided

[9]. Growth hormone has been used in some patients and has shown positive effects, however no clear guidelines on its use have been given [9].

In UCD, medical treatment can 1) replenish deficient substrates, 2) scavenge ammonia, and 3) refill the Krebs cycle by using antioxidants and anaplerotic agents. To replenish deficient substrates L-arginine and L-citrulline can be used. In CPS1-D, OTC-D and HHH syndrome either L-arginine or L-citrulline can be prescribed, while in ASS-D and ASL-D L-citrulline is prescribed. Carbamylglutamate is an effective drug in NAGS-D. Medical treatments that can scavenge ammonia and which are used for this purpose in UCD are sodium benzoate and sodium phenylbutyrate. A known side effect of sodium phenylbutyrate is that it can decrease plasma levels of branched chain amino acids [43]. In UCD patients with a carnitine deficiency L-carnitine should be prescribed.

4.3 | Solid organ transplantation

For OAD, solid organ transplantation of the liver in PA and the liver and/or kidney in MMA has been performed. Liver transplantation can at least partially correct the enzyme deficiency in MMA and PA patients. However, it is not clear when and who to transplant and whether this is a safe treatment option. For UCD, solid liver transplantation is considered curative and has been reported for all UCD except NAGS-D and HHH syndrome. For many classical OAD and UCD patients neurological sequelae have been reported, which were not reversed by the transplantation. Furthermore, for decision making risks related to the transplantation procedure and the use of immunosuppressive medications required after the transplantation need to be taken into account.

5 | Aims and outlines of this thesis

This thesis comprises a total of seven studies supporting the hypothesis that patient's outcome can be improved by optimization of the currently available treatment. To answer this hypothesis we established four study goals.

1. To explore the natural history of classical OAD and UCD patients and the treatments applied, to provide insight in the outcome/prognosis obtained with the currently applied therapies.
2. To determine the most optimal dietary treatment advice.
3. To improve monitoring of classical OAD and UCD patients.
4. To determine the outcome (mortality, complications, quality of life (QOL) and cognitive development) of AOA patients who received solid organ transplantations.

Chapter 2 provides a study in classical OAD and UCD patients on compliance with the current guidelines in daily clinical practice and the outcome of patients achieved with the applied treatment. **Chapter 3** provides insights on how to improve growth in classical OAD and UCD patients. **Chapter 4** describes the effect of dietary treatment on outcome of patients and provides recommendations for improvement of current dietary treatment strategies in MMA and PA patients. **Chapter 5** focuses on the potential role of a biomarker as a predictor of the development of long-term complications in patients and **Chapter 6** provides a study on the potential expected effect of newborn screening in MMA and PA on outcome. **Chapter 7** shows the current practice of solid organ transplantation in patients with AOA in Europe and the neurological risks after transplantation caused by calcineurin inhibitors. In **Chapter 8** the results of the various studies are discussed and conclusions, recommendations and future perspectives are provided.

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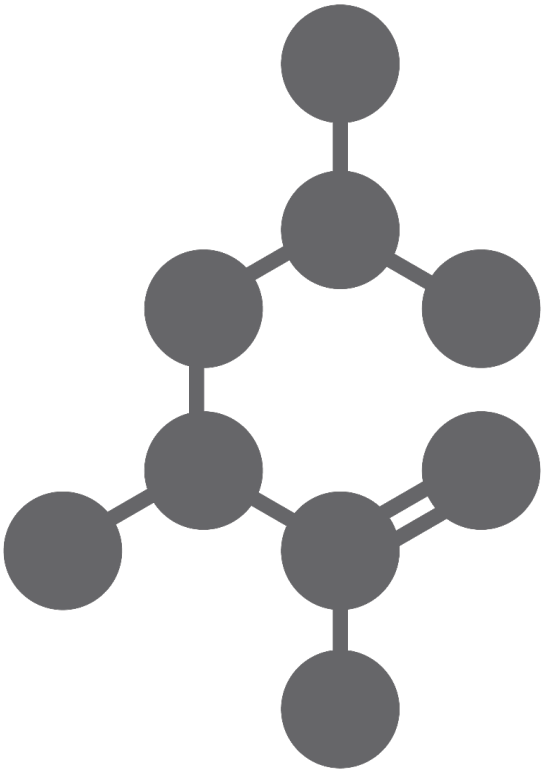

CURRENT TREATMENT APPLIED IN UCD AND CLASSICAL OAD PATIENTS

Evaluation of dietary treatment and amino acid supplementation in organic acidurias and urea-cycle disorders. On the basis of information from a European multicenter registry (E-IMD)

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Abstract

Introduction: Organic acidurias (OAD) and urea-cycle disorders (UCD) are rare inherited disorders affecting amino acid and protein metabolism. As dietary practice varies widely, we assessed their long-term prescribed dietary treatment against published guideline and studied plasma amino acids levels.

Method: We analyzed data from the first visit recorded in the European registry and network for intoxication type metabolic diseases (E-IMD, Chafea no. 2010 12 01).

Results: In total, 271 methylmalonic aciduria (MMA) and propionic aciduria (PA) and 361 UCD patients were included. Median natural protein prescription was consistent with the recommended daily allowance (RDA), plasma L-valine (57%) and L-isoleucine (55%) levels in MMA and PA lay below reference ranges. Plasma levels were particularly low in patients who received amino acid mixtures (AAMs-OAD) and L-isoleucine: L-leucine: L-valine (BCAA) ratio was 1.0:3.0:3.2. In UCD patients, plasma L-valine, L-isoleucine and L-leucine levels lay below reference ranges in 18%, 30% and 31%, respectively. In symptomatic UCD patients who received AAM-UCD, the median natural protein prescription lay below RDA, while their L-valine and L-isoleucine levels and plasma BCAA ratios were comparable to those in patients who did not receive AAM-UCD. Notably, in patients with ornithine transcarbamylase syndrome (OTC-D), carbamylphosphate synthetase 1 syndrome (CPS1-D) and hyperammonemia-hyperornithinemia-homocitrullinemia (HHH) syndrome selective L-citrulline supplementation resulted in higher plasma L-arginine levels than selective L-arginine supplementation.

Conclusion: While MMA and PA patients who received AAMs-OAD had very low BCAA levels and disturbed plasma BCAA ratios, AAMs-UCD seemed to help UCD patients obtain normal BCAA levels. In patients with OTC-D, CPS1-D and HHH syndrome, selective L-citrulline seemed preferable to selective L-arginine supplementation.

1 | Introduction

Organic acidurias (OAD) and urea-cycle disorders (UCD) are rare inherited disorders affecting amino acid and protein metabolism. Their estimated incidence is 1 in 1,000,000–50,000 newborns per individual disease [1, 2]. OAD (methylmalonic aciduria (MMA) (mutase deficiency OMIM #251000, CblA #251100, CblB #251110) and propionic aciduria (PA) (OMIM #606054)) are caused by deficiencies of the enzymes needed for the breakdown of branched-chain amino acids (BCAA), L-valine and L-isoleucine in MMA and PA. UCD, i.e. inherited deficiency of N-acetylglutamate synthase (OMIM #237310), carbamylphosphate synthetase 1 deficiency (CPS1-D) (EC 6.3.4.16; OMIM #237300), ornithine transcarbamylase deficiency (OTC-D) (EC 2.1.3.3; OMIM #311250), argininosuccinate synthetase deficiency (ASS-D) (EC 6.3.4.5; OMIM #215700), argininosuccinate lyase deficiency (ASL-D) (EC 4.3.2.1; OMIM #207900), arginase 1 deficiency (OMIM #207800) and hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM #238970), are caused by deficiencies of the enzymes and transporter needed for the urea-cycle. If left untreated, ammonium and other toxic metabolites accumulate.

In OAD patients long-term treatment consists of reducing natural protein intake to prevent intoxication while allowing normal growth [3]. Although, when necessary, precursor-free amino acid mixtures (AAM) (AAMs-OAD: amino acid mixtures in OAD) can be supplemented without inducing intoxication, there has recently been some debate on their use with respect to the relative high L-leucine content influencing other plasma BCAA levels [3-6]. In MMA and PA, AAMs-OAD lack L-valine and L-isoleucine. In some patients, single amino acids (SAA) can be supplied to fine-tune dietary treatment, though there is no international consensus on the use of either AAMs-OAD or SAA [7]. In UCD patients, the purpose of dietary treatment is to reduce the nitrogen load. In the most severe cases, treatment consists of a protein-restricted diet (either with or without the use of AAM-UCD (amino acid mixture in UCD)), supplemented with L-arginine and/or L-citrulline to support the urea-cycle [8]. Most patients also receive nitrogen scavengers. In patients with mild phenotypes, treatment can be less strict, ranging from a self-initiated vegetarian diet to a natural protein restriction without nitrogen scavengers and other supplements. In both OAD and UCD, additional caloric intake to maintain anabolism is necessary [9].

In daily practice, dietary treatment is currently very diverse [4, 10]. If long-term outcome in these patients is to be improved, it is essential that treatment is optimized. The guideline proposed by Baumgartner et al for the dietary treatment of patients with MMA and PA [11] suggest that "the FAO/WHO/UNU (2007) safe levels of protein intake are a useful guide for protein prescription". The guideline suggests that "synthetic protein should form part of the total protein intake if natural protein tolerance is below FAO/WHO/UNU (2007) safe levels" [11], and the Genetic Metabolic Dietitian International guideline on PA recommends that AAMs-OAD (which lack L-isoleucine and L-valine) should be added to achieve 100-120% of recommended daily allowance (RDA) in those patients who tolerate a natural protein intake less than 100% RDA [12]. However, there is no guideline available on the amount of synthetic protein intake as percentage of total protein intake in those patients requiring supplemental amino acids. The guideline for UCD proposed by Häberle et al [13] likewise suggests that the FAO/WHO/UNU requirements are used for protein intake. The guideline provides recommendations on the amount of synthetic protein intake as a percentage of the total protein intake for patients who require supplemental amino acids (recommendation: 20-30%). The same guideline also propose recommendations on dosages of L-citrulline and/or L-arginine treatment (L-arginine dose: <20kg body weight: 100-200mg/kg/day, >20kg body weight: 2.5-5g/m^2/day and L-citrulline dose: 100-200mg/kg/day). However, these dietary and supplemental recommendations are based on expert opinion. There are neither outcome data with respect to recommendations on appropriate plasma amino acid levels, nor are there clear recommendations on whether L-citrulline or L-arginine treatment is preferred in any of the following: CPS1-D, OTC-D and HHH syndrome.

If optimized treatment is feasible, it is likely to improve outcome in OAD and UCD patients. To establish whether or not treatment is sufficient, it is very important to evaluate dietary and supplemental treatment in a large cohort of OAD and UCD patients. As the first step in this evaluation, we evaluated data from the first visit recorded in the registry from OAD and UCD patients in the European registry and network for Intoxication type Metabolic Diseases (E-IMD) 1) to compare their current long-term dietary and supplemental treatment with the existing guideline; and 2) to study the plasma amino acids levels in patients with this prescribed treatment.

2 | Methods

2.1 | Patient registry and inclusion/exclusion criteria

The European registry and network for Intoxication type Metabolic Diseases (E-IMD; URLs: http://www.e-imd.org (website); https://www.eimd-registry.org (registry)) is a European project that was initiated in 2011 and now includes a web-based patient registry containing comprehensive follow-up data on more than 1,200 individuals with OAD and UCD. A detailed overview of the E-IMD was published previously [14]. The data in the E-IMD registry were entered by clinicians and dieticians. The dietary information in this registry was the diet prescribed, which does not necessarily equal the consumed diet. The study was approved by the local ethics committee of the coordinating center (University Hospital Heidelberg) and by all clinical partners. The current publication project was evaluated by the scientific board and approved by the executive board of the E-IMD consortium.

In this cross-sectional study, we included all data from the first visits recorded in the registry and all visits included were during stable metabolic period. We included MMA (mut-, mut0, cblA, and cblB), PA, OTC-D females and males, CPS1-D, HHH syndrome, ASS-D and ASL-D patients. CblA and CblB patients were included as the majority of them were symptomatic and they were also treated with a natural protein restriction. We excluded patients with other inherited metabolic diseases, those with an unconfirmed suspicion of an OAD or UCD, those with MMA CblC and CblD subtypes and unclassified type, and those who had received a kidney or liver transplantation or had other serious unrelated comorbidities, such as Down syndrome, kernicterus, or fetal alcohol syndrome. We furthermore excluded those without information on the prescribed dietary treatment and those without information on clinical symptoms (symptomatic or not). Clinically symptomatic patients were defined as presenting with a metabolic crisis or long-term complications.

2.2 | Data analysis

Synthetic protein was defined as a protein-equivalent of specialized AAM-OAD/UCD and SAA. L-valine and/or L-isoleucine were supplied independently or in combination as SAA. Total protein prescription was calculated by adding natural protein and synthetic protein

prescription. Protein prescription (natural, synthetic and total) data from the first visit recorded in the registry was compared with the WHO RDA [15]. Synthetic versus total protein prescription (ratio) was calculated by dividing the amount of synthetic protein % RDA by total protein % RDA. Information on the amino-acid content of AAMs-OAD/UCD was obtained from the manufacturers. Supplementation with L-arginine and/or L-citrulline and the provision of sodium phenylbutyrate were both compared with current recommendations [13]. Plasma amino acid levels were compared with the amino acid reference values provided in table 2.1.5 of the "Laboratory Guide to the Methods in Biochemical Genetics" by Duran et al. 2008 [16].

On the basis of their common metabolic amino acid disturbances, we combined the following disorders in several analyses: 1) MMA and PA; 2) inherited deficiency of ASS-D, ASL-D; 3) OTC-D males, CPS1-D and HHH syndrome and 4) as a separate group OTC-D females.

2.3| Statistical analysis

SPSS (IBM SPSS Statistics 22.0) was used for descriptive statistics (percentages, mean, standard deviation, median and range). Normality was examined using the Kolmogorov-Smirnov test and quantile-quantile (Q-Q) plots. We corrected the p-values per outcome for the subgroup analyses by using the Holm method. Student's t-test was performed to compare means if distribution was Gaussian and Wilcoxon rank sum test (Ws) if distribution was non-normal. If there were dichotomous parameters in a small sample size Fisher's exact test was performed to determine statistical significance between groups and if there were dichotomous parameters in a large sample size chi square test was performed. One-way ANOVA was performed to compare more than two groups. To compare L-citrulline and L-arginine prescription against the guideline, body-surface area was calculated with the Mosteller formula. To identify variables that had significant associations with plasma amino acid levels, we used backward stepwise linear regression analysis to explain the relationship between five independent variables on plasma amino acids. As forward methods produce suppressor effects, we performed backward linear regression [17]. Five independent variables

were used in the multiple regression analysis: protein prescription (natural (% RDA) and synthetic); use of single branched-chain amino acids (yes/no); age at visit; clinically symptomatic (yes/no); and, in UCD, use of sodium phenylbutyrate (yes/no). On the basis of the amount of L-arginine/L-citrulline prescription and of selective L-citrulline versus L-arginine supplementation, we also used backward stepwise linear regression to identify factors that had an association with plasma L-arginine levels in OTC-D and CPS1-D and HHH syndrome.

3 | Results

3.1 | Description of the study population

Two hundred and seventy-one MMA and PA patients and 361 UCD patients had been registered between 1 February 2011 and 20 May 2016 (supplementary table 1, 4). In patients with MMA and PA as well as in patients with UCD, OTC-D females excluded, 82% was symptomatic. A total of 67% of females with OTC-D were symptomatic. In total, 73% of patients with OAD and UCD were diagnosed by selective metabolic testing, 13% by newborn screening, 13% by high-risk family or population screening, and 1% by prenatal testing. Most patients were from Europe (95%), the remainder from Taiwan (2%), the USA (2%), India (0.8%) and Japan (0.2%). Median age at first visit recorded in the registry was 9.3 years (5-95% percentile: 0.4 – 35.6). The median time from diagnosis until the first visit recorded in the registry was 6.6 years (5-95% percentile: 0.2 – 23.4). Information on participating countries and disease groups is provided in supplementary table 2.

3.2 | MMA and PA

3.2.1 | MMA, PA and protein prescription

A total of 92% (250/271) MMA, PA patients received a protein restricted diet (supplementary figure 1). Natural protein prescription was according to and above the RDA in 62%. Symptomatic MMA, PA patients received a lower natural and total protein prescription %

RDA than asymptomatic patients (supplementary table 3a, figure 1a). A few patients were prescribed a natural protein intake <50% or >200% RDA (figure 1a). Various (29) AAMs-OAD were provided by a total of six companies and used for a majority of MMA and PA patients (supplementary figure 1). AAMs-OAD were free of L-valine and L-isoleucine, while the rest of their content varied (supplementary table 4). Median protein prescription through AAMs-OAD was 0.50 g/kg/d (5-95% percentile: 0.18- 1.20). SAA were supplied in only 17% of those with MMA (24/144) and 20% of those with PA (25/127). Ninety percent of those who received SAA (44/49) were also prescribed AAM-OAD. Natural protein prescription was according to and above the RDA in the majority (58%, 96/166) of patients who received synthetic protein (figure 2, supplementary table 3a). The mean amounts of synthetic protein as a percentage of total protein prescription was 40% (SD ±15.2). In patients with MMA, those with the Mut^0 subtype were prescribed the lowest natural protein % RDA. AAMs-OAD were most frequently used in Mut0 and CblB patients (supplementary table 5).

Protein prescription within the different countries varied, with a median natural protein prescription of 112% RDA, a total protein prescription of 159% RDA and a synthetic versus total protein prescription of 40% (supplementary table 2). A high natural protein prescription was seen in the Austria (n=12), Taiwan (n=7) and Romania (n=2) and the highest total protein prescription in Czech Republic (n=10), Spain (n=20), Romania (n=2), the USA (n=10) and Japan (n=1). The synthetic versus total protein prescription was highest in UK (n=1), Japan (n=1), Denmark (n=4), Spain (n=13) and Portugal (n=2) (supplementary table 2). Compared to the other countries, AAM-OAD was not applied in Serbia and Taiwan.

3.2.2 | Impact of dietary management on plasma amino acids in MMA, PA

Plasma BCAA levels were reported in 66% (180/271) of the MMA and PA patients (supplementary table 1). In individuals with MMA and PA plasma L-valine and L-isoleucine levels both lay below the lowest reference ranges in 57% and 55% of the patients respectively (figure 3a). Symptomatic MMA and PA patients who were prescribed AAM-OAD had lower plasma L-valine and L-isoleucine levels than those without (figure 4, supplementary table 3a). Linear regression analysis showed that the plasma L-valine was positively associated with the amount of natural protein prescription % RDA and negatively

associated with the amount of L-leucine prescription derived from AAM-OAD (supplementary table 3c). Plasma amino acid levels of L-isoleucine, L-valine and L-leucine were the lowest in patients with Mut0 and cblB phenotype (supplementary table 5). In MMA and PA median plasma L-isoleucine: L-leucine: L-valine ratio was 1: 2.5: 2.9 (reference value: 1: 2: 4). Patients with AAM-OAD had ratio of 1: 3.0: 3.2, while those without AAM-OAD had ratio of 1: 1.9: 3.3. The ratio of L-leucine versus L-isoleucine plasma levels (Ws=4088.0, Z=-4.590, $p<0.001$) as well as the ratio of L-leucine versus L-valine plasma levels (Ws=3569.5, Z=-6.084, $p<0.001$) were significantly higher in patients who received AAM-OAD compared to those without.

3.3 | Urea-cycle disorders

3.3.1 | UCD and protein prescription

A total of 80% (289/361) of the UCD patients received a protein restricted diet (supplementary figure 1). Median natural protein prescription in symptomatic and asymptomatic patients was close to or above the RDA; in the majority of UCD subgroups, total protein prescription was above the RDA (figure 1a-d). Several patients were prescribed a natural protein intake protein <50% or >200% RDA (figure 1b-d). For UCD 21 different AAMs-UCD were provided by a total of 5 companies. AAMs-UCD were supplemented with BCAA (L-valine, L-isoleucine and L-leucine) (supplementary table 4). AAMs-UCD were used for 32% (114/361) of the patients (supplementary figure 1) and median protein prescription through AAM-UCD was 0.28 g/kg/d (range: 0.04-1.17 g/kg/d).

Symptomatic patients who received AAM-UCD had a lower natural protein % RDA prescribed than those who did not receive AAM-UCD in the CPS1-D, OTC-D male and HHH-syndrome subgroup (supplementary table 3b). In the ASS-D, ASL-D and CPS1-D, OTC-D male and HHH-syndrome subgroups, natural protein prescription in symptomatic patients taking AAM-UCD lay below the RDA (figure 2). SAA of BCAA were supplied in only 3% (11/361); 3/11 of those who received SAA were also prescribed AAM-UCD. The mean amounts of synthetic protein as a percentage of total protein prescription were as follows in the

following groups: 31% in the ASS-D and ASL-D subgroup (SD ±18.2), 32% in the CPS1-D, OTCD-male and HHH subgroup (SD ±11.7) and 28% (SD ±14.2) in OTC-D females.

Protein prescription within the different countries varied, there was a high median natural protein prescription in Poland (n=9), Denmark (n=10) and India (n=4), while a low median natural protein prescription in Austria (n=3), Italy (n=39) and UK (n=8). Austria and the UK had a relatively high synthetic versus total protein prescription and thereby a total protein prescription according to recommendations, while Italy had a low total protein prescription. AAMs-UCD were prescribed in the majority of patients in the Netherlands, Austria and Greece, Czech Republic, while in the minority in all other countries (supplementary table 2).

FIGURE 1. Protein prescription in patients with MMA and PA (A); patients with ASS-D or ASL-D (B); patients with CPS1-D, males with OTC-D or patients with HHH syndrome (C); and in females with OTC-D (D).

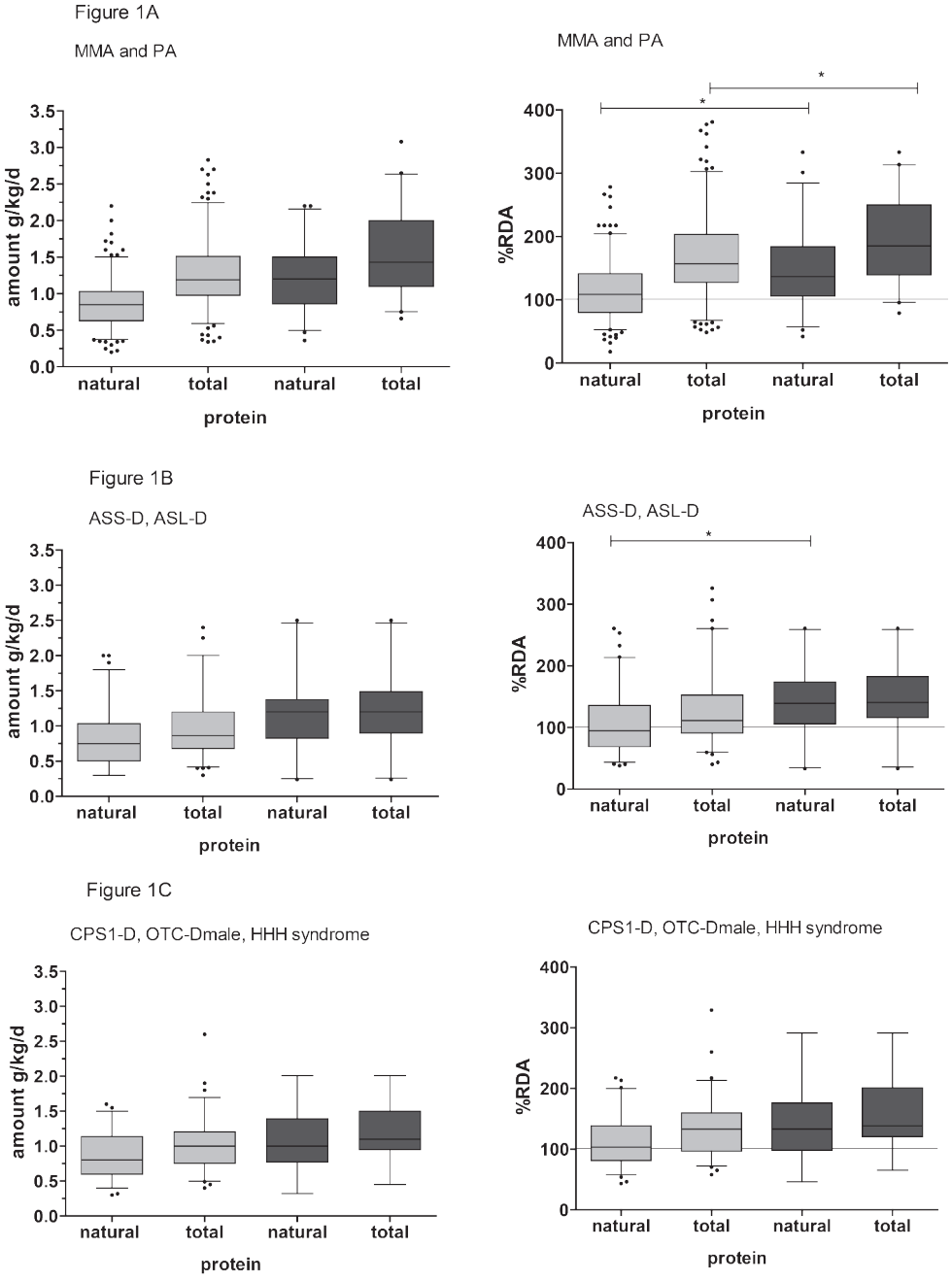
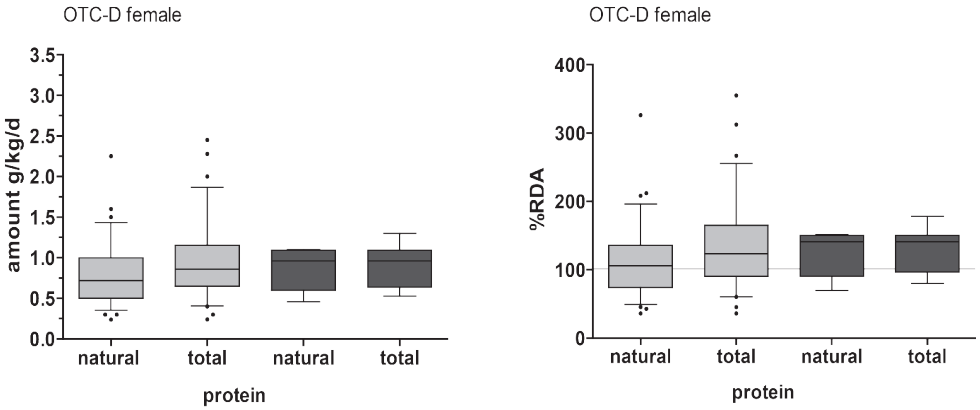


Figure 1D



Gray boxes indicate symptomatic patients and black boxes indicate asymptomatic patients. Synthetic protein indicates the amounts only for patients receiving synthetic protein. Circles: outliers; whiskers: 5-95 percentile; horizontal line: median; * $p<0.05$

3.3.2 | Sodium phenylbutyrate in UCD

Sodium phenylbutyrate was provided in 37% of the UCD patients (134/361). In the CPS1-D, OTC-D male and HHH-syndrome subgroup, prescribed natural protein and total protein % RDA was lower in those prescribed sodium phenylbutyrate treatment than in those who were not (supplementary table 3b). Sodium phenylbutyrate was applied in the majority of patients in Germany, Netherlands, Croatia, Czech Republic and Taiwan (supplementary table 2d).

3.3.3 | Impact of dietary management and sodium phenylbutyrate on branched chain amino acids in UCD

In 76% of UCD patients (277/361) BCAA levels were reported (supplementary table 1). In total UCD, plasma L-valine, L-isoleucine and L-leucine levels lay below the levels of reference values in 18%, 30% and 31% of the patients, respectively (figure 3b-d). Supplementary table 3b shows plasma BCAA levels of the ASS-D, ASL-D subgroup, OTC-D male, CPS1-D, HHH syndrome subgroup and OTC-D female subgroup in symptomatic versus asymptomatic

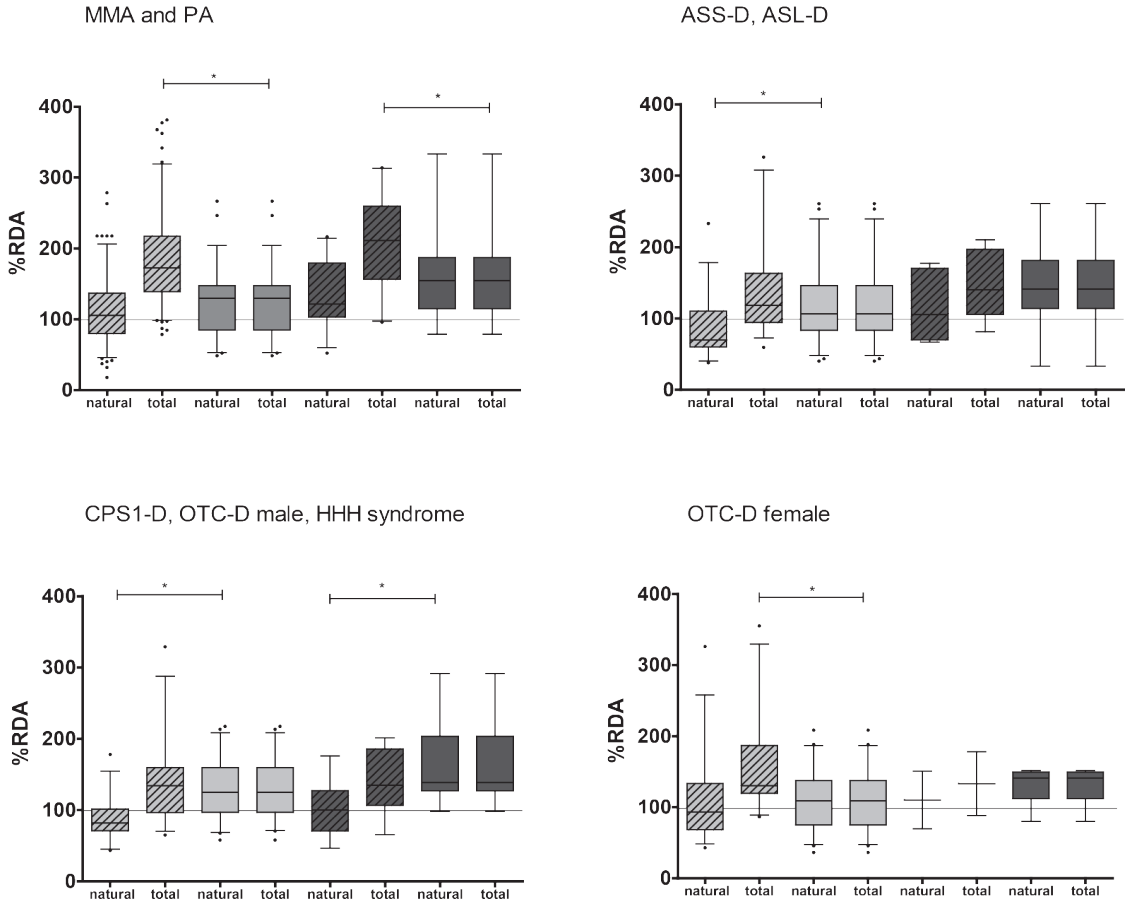
patients and in those with a natural protein restricted diet versus those without. In these UCD subgroups, plasma BCAA levels in patients who received AAM-UCD did not differ from those who did not receive AAM-UCD (supplementary table 3b). In total UCD patients, linear regression analysis showed that plasma L-valine, L-isoleucine and L-leucine were associated with sodium phenylbutyrate treatment and age at visit (supplementary table 3d). The UCD patients had a plasma L-isoleucine: L-leucine: L-valine ratio of 1: 1.7: 3.7 (reference value: 1: 2: 4). Patients who received AAM-UCD had a ratio of 1: 1.7: 3.7 and those who did not receive AAM-UCD had ratio of 1: 1.9: 3.7.

3.3.4 | L-arginine and/or L-citrulline treatment in UCD

L-arginine was provided in most ASS-D and ASL-D patients, i.e., in 95% of the symptomatic patients (90/95) and 86% of the asymptomatic patients (18/21). In 31% of ASS-D (18/58) and 31% of the ASL-D patients (14/45), L-arginine-supplemented doses were above the maximum recommended guideline (6 g/day [13]). Individuals who received L-arginine in the ASS-D and ASL-D subgroup had lower natural protein % RDA and total protein % RDA prescription than those who did not receive L-arginine treatment (supplementary table 3b).

In the CPS1-D, OTC-D male and HHH-syndrome subgroup, 86% of the symptomatic patients (82/95) and 59% of the asymptomatic patients (13/22) were prescribed L-citrulline and/or L-arginine. One or both of these amino acids were also prescribed in 79% of the symptomatic females with OTC-D (68/86) and in 21% of the asymptomatic females (9/42). In those who received L-citrulline and/or L-arginine, selective supplementation with L-citrulline was provided in 44% (76/172); a combination of L-citrulline and L-arginine was provided in 20% (34/172); and selective L-arginine supplementation was provided in 36% (62/172). Many patients were prescribed L-citrulline (44% (48/109)) and L-arginine (24% (22/92)) above recommended dose [13].

FIGURE 2. Protein prescription and the use of AAM.



Grey boxes indicate symptomatic patients and black boxes indicate asymptomatic patients. Diagonally striped boxes indicate patients in whom AAM was prescribed, and boxes without stripes indicate patients without AAM. Circles: outliers; whiskers: 5-95 percentile; horizontal line: median; * $p<0.05$

FIGURE 3. Plasma L-valine, L-isoleucine, L-leucine levels in patients with MMA and PA (A); patients with ASS-D or ASL-D (B); patients with CPS1-D, males with OTC-D or patients with HHH syndrome (C); and in females with OTC-D (D).

Figure 3A

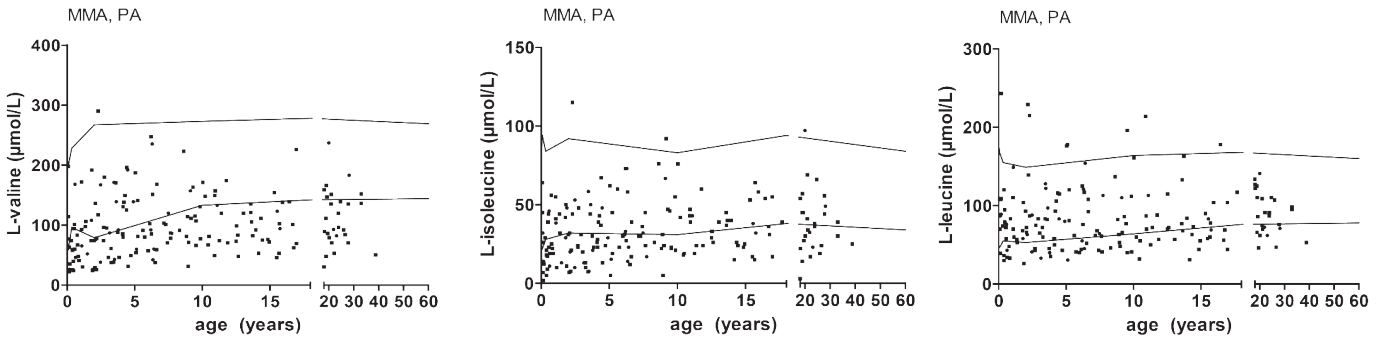


Figure 3B

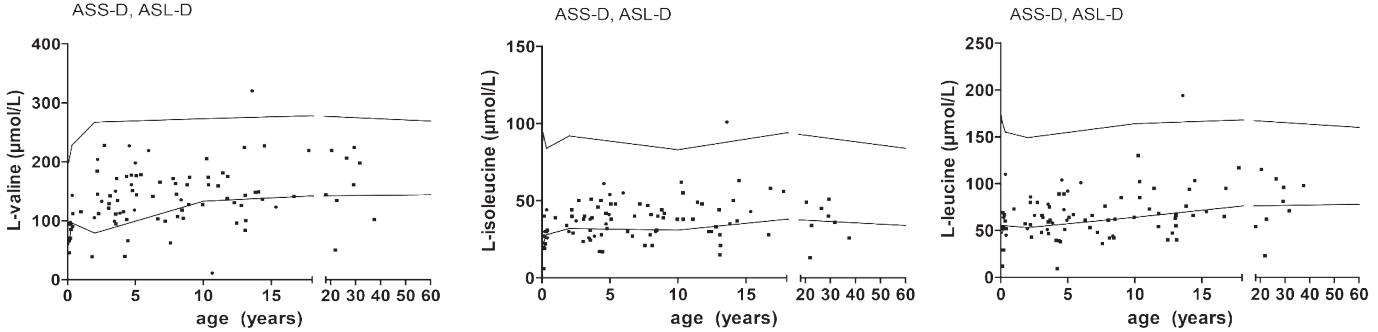


Figure 3C

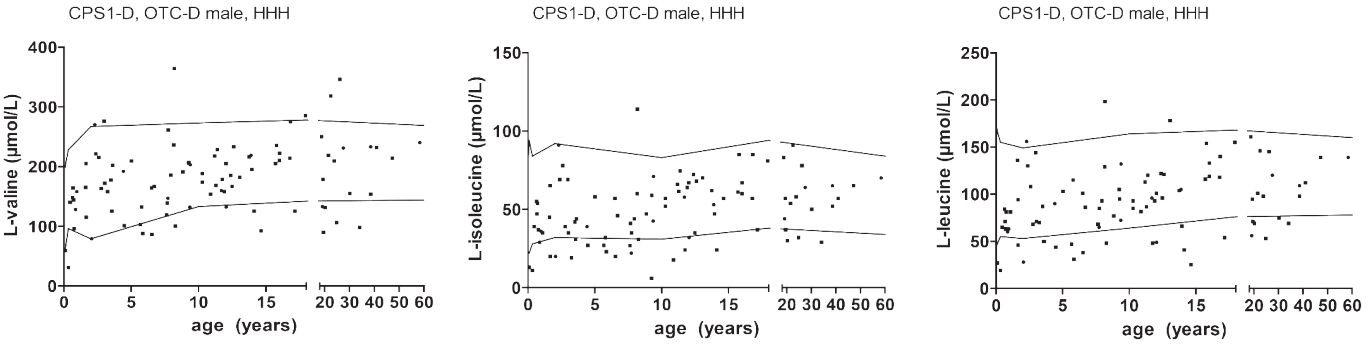
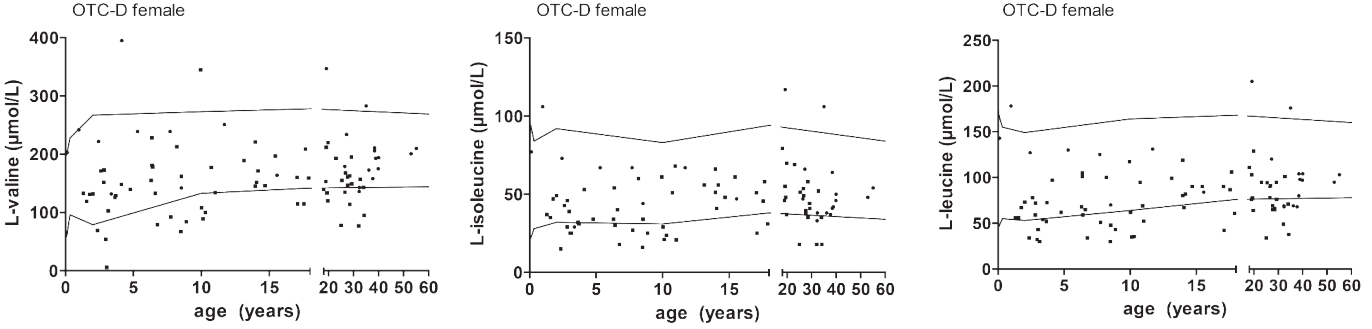


Figure 3D

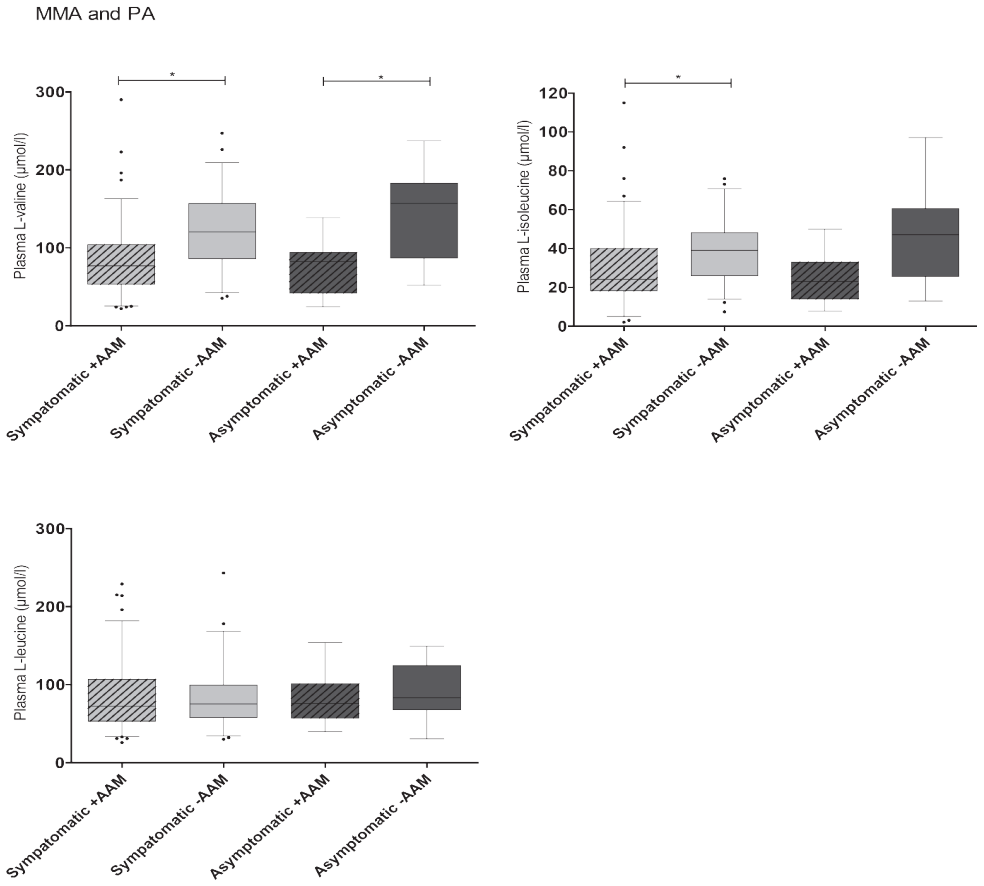


Horizontal lines indicate upper and lower levels of reference values [16]. Each point represents a single measurement for a particular patient.

3.3.5 | UCD and levels of plasma L-arginine and L-citrulline

In the subgroup of patients with CPS1-D, OTC-D (male and female) and HHH syndrome, patients who were prescribed selective L-citrulline supplementation had higher plasma L-arginine levels than those who were prescribed only L-arginine (Ws=2745.5, Z= -3.066, p=0.002) and they had higher plasma L-arginine levels than those without supplementation (Ws=1406.0, Z=-5.109, p<0.001) (supplementary figure 2). Plasma L-arginine levels in patients who received L-citrulline only did not differ from those in patients who received a combination of L-citrulline and L-arginine (supplementary figure 2). The difference in plasma L-arginine levels between symptomatic and asymptomatic patients can be found in supplementary table 3b. Plasma L-arginine levels did not differ between OTC-D males and females. The ratio of OTC-D male versus female did not differ within patients receiving either L-citrulline or L-arginine or a combination of these two. Patients (CPS1-D, OTC-D (male and female) and HHH syndrome) who received either L-citrulline, L-arginine or a combination of both did not differ with regard to the age at visit, prescription of natural protein % RDA and prescription of protein derived from AAM-UCD. However, the mean L-arginine prescription (mg/kg/day) in patients who received L-arginine was lower than the mean L-citrulline prescription (mg/kg/day) in patients who received L-citrulline (Ws=3397.0, Z=-2.518, p=0.012). Regression analysis showed that plasma L-arginine levels were associated with selective L-citrulline therapy versus L-arginine monotherapy (β-coefficient =0.250, p=0.007) (R^2 =0.062, $R^2_{Adjusted}$= 0.054), while the dose of either L-arginine or L-citrulline was not significantly associated with plasma L-arginine levels in the regression analysis.

FIGURE 4. Plasma L-valine, L-isoleucine and L-leucine levels in patients with MMA and PA.



Grey boxes indicate symptomatic patients and black boxes indicate asymptomatic patients. Diagonally striped boxes indicate patients in whom AAM was prescribed, and boxes without stripes indicate patients without AAM. Reference values are age dependent and can been found in figure 3 (A).

4 | Discussion

The purpose of this cross-sectional study was to evaluate current prescribed long-term dietary and supplemental treatment in OAD and UCD. We used the E-IMD registry to survey dietary management approaches of 271 OAD and 361 UCD patients to compare their current long-term dietary and supplemental treatment with the existing guideline; and to study the plasma amino acids levels in a total of 457 patients with this prescribed treatment. It is essential to evaluate this treatment, not only because long-term outcome in OAD and UCD continues to be disappointing, but also because newborn screening for these diseases is upcoming in an increasing number of countries.

We have three main findings. First, plasma L-valine and L-isoleucine levels were very low in most patients with MMA and PA, mainly in symptomatic patients who received AAM-OAD (which lack L-valine and L-isoleucine), while median daily natural protein prescription was consistent with the current RDA. The high L-leucine content in AAM-OAD seemed to affect the plasma L-valine levels and lead to abnormal ratios of plasma L-leucine: L-isoleucine and L-leucine: L-valine.

Second, of patients with UCD plasma BCAA levels lay below reference ranges in approximately 20-30%. While natural protein prescription lay below the RDA in most symptomatic patients who received AAM-UCD, plasma L-valine and L-isoleucine levels and L-isoleucine: L-leucine: L-valine ratio were similar to those in patients who did not receive AAM-UCD (which contain essential amino acids). Sodium phenylbutyrate was correlated with low BCAA levels as previously reported [18, 19]. Third, plasma L-arginine levels were significantly higher in patients with CPS1-D, OTC-D and HHH syndrome who were prescribed selective L-citrulline supplementation than in patients who were prescribed no supplementation or selective L-arginine supplementation.

Some limitations of this study and of the registry should be addressed. First, as no diary-based data was available on protein intake and there was no data on the patients' adherence to dietary treatment, we were unable to analyze actual dietary intake and neither total branched chain amino acid intake. Furthermore, quality of protein consumed was not known.

In this study we assumed that the patients took all the prescribed amounts. The large number of patients included in this study can give general information. As a next step actual dietary intake and intake of branched chain amino acids and its effect should be evaluated. Second, in this study kilocalorie intake was not incorporated and should be further studied in upcoming studies. Third, there was a high variability in the AAMs-OAD/UCD prescribed and they should be studied in more detail to specifically determine their effects. Fourth, the quantitative amino acid analyses were only a single measurement and not all patients included had BCAA levels measured. Our cross-sectional approach did not allow easy identification of predictors of plasma amino acid levels, it is difficult to assign a cause-effect relationship. Fifth, due to the high number of participating centers plasma amino acids levels are determined by various methods and different reference values are used, which can have impact on the conclusions drawn. No quality checks were in place to ensure the accuracy of data collected at each site. We did not assess the correctness of measurements and the differences between contributing laboratories. However, most laboratories participate regularly in external quality assessment (ERNDIM). Furthermore, the registry does not record the interval between the last intake and sampling of amino-acid plasma levels. However, as compartmentalization of plasma amino acids can affect the interpretation of plasma amino acid levels, samples should be taken accurately between 3.5 and 4 hours after the last meal [20]. Last, there can be a bias in the patients included, since some countries have high numbers of OAD and UCD, but this is not reflected in the E-IMD registry. All the limitations specified here require attention in future studies.

4.1 | MMA, PA: protein prescription, AAM-OAD and plasma amino acid levels

Our results show that while natural protein prescription was often close to the RDA and total protein prescription was even above the RDA, with current dietary prescription the majority of patients had plasma BCAA concentrations below the reference ranges. The low BCAA levels in MMA and PA were previously reported by others [4, 5, 21, 22] as well as the observation that daily natural protein prescription was according to the current RDA in MMA and PA [23, 24]. Towards improving patient care, it thus seems necessary to fine-tune plasma BCAA levels in individual patients. BCAAs are essential for keeping up anabolism and a

decrease in plasma BCAA concentrations can herald an acute metabolic crisis [18]. Furthermore, BCAAs are essential for supporting normal growth and development [25-27].

Since a low natural protein intake can be potentially harmful, i.e. low BCAA levels, one should ensure that each patient achieves a higher natural protein intake in a way that does not cause metabolic instability. Protein prescription is currently based on the recommendations provided by the WHO 2007 [15]. While these recommendations are based on individuals consuming protein of a high biological value, the proteins commonly used by OAD patients are not only of low biological value, but are also poorly digestible [24]. Consequently, the WHO 2007 recommendation does not seem applicable to these patients, an appropriate guideline for their protein prescription is necessary. However, due to interpatient variability this may be hard to achieve.

In OAD, AAM products are free of L-isoleucine, L-methionine, L-threonine, and L-valine, since these amino acids are precursors of toxic metabolites. While the guideline suggests prescribing AAM-OAD in those patients with a natural protein prescription below the RDA, we observed that the majority of those who received AAM-OAD had natural protein prescription that was according to and above the RDA. Nevertheless, our results show significantly lower plasma L-valine and L-isoleucine levels in symptomatic patients who received AAM-OAD. Those who received AAM-OAD are; in view of the very low L-valine and L-isoleucine levels, likely to be at risk of decompensations and growth retardation, and potentially of long-term complications. It is noteworthy that, in a small cohort of MMA patients, a high intake of L-leucine derived from AAM-OAD was associated with lower L-valine and L-isoleucine plasma levels [5]. This could possibly be explained by means of the competitive interaction of BCAA on the same receptors (such as the large neutral amino acid transporter, LAT1[28]). We confirm this inverse relationship between L-leucine intake derived from AAM-OAD and plasma L-valine levels in our large patient cohort (of MMA and PA patients) with correction for covariates such as natural protein prescription. We furthermore showed that L-leucine: L-isoleucine as well as L-leucine: L-valine ratios were higher in those who received AAM-OAD versus those who did not, which is in line with a study by Myles et al. [6]. In patients whose natural protein intake cannot be raised, L-valine and L-isoleucine plasma levels may be optimized by reducing the L-leucine content of AAM-OAD. However, as the coefficients of the negative correlation between L-leucine derived from AAM-OAD and

plasma L-valine and L-isoleucine levels were small, it is possible that such a reduction will be effective only in a small number of patients. It may also be hazardous: although our data do not confirm this, further reducing the daily L-leucine intake might lead to even lower L-leucine plasma levels than found (low plasma L-leucine levels were previously reported [21, 23, 29]), and thereby increase the risk of catabolism. In conclusion, AAMs-OAD for MMA and PA patients should be prescribed with care and with full awareness of their potentially harmful consequences [6].

In MMA and PA patients supplementation with SAA can play a role in dietary treatment [7]. In the overall group of MMA, PA patients, the L-isoleucine: L-leucine: L-valine ratio was 1: 2.5: 3.0 and in those patients without AAM-OAD ratio was 1: 1.9: 3.3, which indicates low L-valine levels (normal L-isoleucine: L-leucine: L-valine ratio is 1: 2: 4 [16, 28]). The disturbed BCAA ratios in patients without AAM-OAD could be due to the low biological value of protein consumed. In patients where maximal protein tolerance has been reached, SAA might be an option. In our opinion, calculating BCAA ratios can give an indication whether or not to increase natural protein or to supply SAA.

The first step towards better monitoring of OAD patients was provided by the guideline that recommend monitoring quantitative amino acids every 3-6 months [11]. As it is very difficult to obtain optimal plasma BCAA levels that stimulate growth and development without inducing toxicity, we suggest that individualized patient care might be optimized by more frequent monitoring of BCAAs plasma levels.

4.2 | UCD: protein prescription, AAM-UCD and plasma amino acid levels

Natural protein prescription was often close to the RDA and total protein prescription was even above the RDA in UCD patients. This could be due to the fact that in this study we looked into protein prescription, which is not necessarily equal to the intake. Interestingly, several patients were prescribed high natural protein intake and total protein intake (>200% RDA), which is highly important to be aware of since this puts the patients at risk for hyperammonemia and renal disease. The risk for low plasma BCAA levels is highest in those who received sodium phenylbutyrate [18, 19, 30, 31]. In UCD patients, AAMs-UCD are

supplemented with L-valine, L-isoleucine and L-leucine. We found that the natural protein prescribed in symptomatic UCD patients who received AAM-UCD was lower than recommended, and significantly lower than that in patients without AAM-UCD. Due to AAM-UCD (median dose 0.28 g/kg/day) total protein prescription was consistent with recommendations. We observed that UCD patients who received AAM-UCD —and thus a lower natural protein prescription — achieved plasma L-isoleucine and L-valine levels and L-isoleucine: L-leucine: L-valine ratios similar to patients without AAM-UCD. This suggests that AAMs-UCD have a beneficial effect in UCD in stable disease period.

4.3 | UCD: L-arginine and/or L-citrulline

To date there has been no clear-cut evidence that the efficacy of L-citrulline is greater than that of L-arginine for OTC-D, CPS1-D and HHH syndrome [13]. Now, for the first time, we show that patients who received L-citrulline had higher plasma L-arginine levels than those who received L-arginine alone. Plasma L-arginine levels depend on the amount of L-citrulline and/or L-arginine prescription. The bioavailability of L-citrulline is greater than that of L-arginine [32, 33], and supplementation with L-citrulline leads to higher plasma L-arginine concentrations than supplementation with L-arginine. This supports the notion that it is preferable to use L-citrulline for patients with CPS1-D, OTC-D and HHH syndrome. Importantly, L-citrulline is more expensive than L-arginine [13].

In this study we surveyed long-term prescribed dietary treatment and amino acid supplementation in OAD and UCD patients in the E-IMD registry, taking the limitations of this study into consideration, with the aim to improve treatment. In future studies the possible harmful consequences (i.e., the number of decompensations, growth, long-term complications and mortality) of the very low plasma BCAA levels in MMA, PA and UCD patients must be evaluated. Recommendations on adequate plasma levels in OAD and UCD should be formulated and the efficacy of adjusted treatment (including AAM and/or SAA), without inducing toxicity, needs to be followed.

5 | Conclusion

Current dietary practice in OAD and UCD patients differ widely. Natural protein prescription was close to the RDA, but very low BCAA levels and abnormal BCAA plasma ratios in patients with MMA and PA who were prescribed AAMs-OAD were observed. UCD patients with a risk of low plasma BCAA levels seemed to benefit from BCAA-supplemented AAMs-UCD. Patients with OTC-D, CPS1-D and HHH syndrome who received selective L-citrulline supplementation had significantly higher L-arginine plasma levels than patients who received no supplementation or selective L-arginine. These results make it possible to further improve recent treatment recommendations for OAD and UCD.

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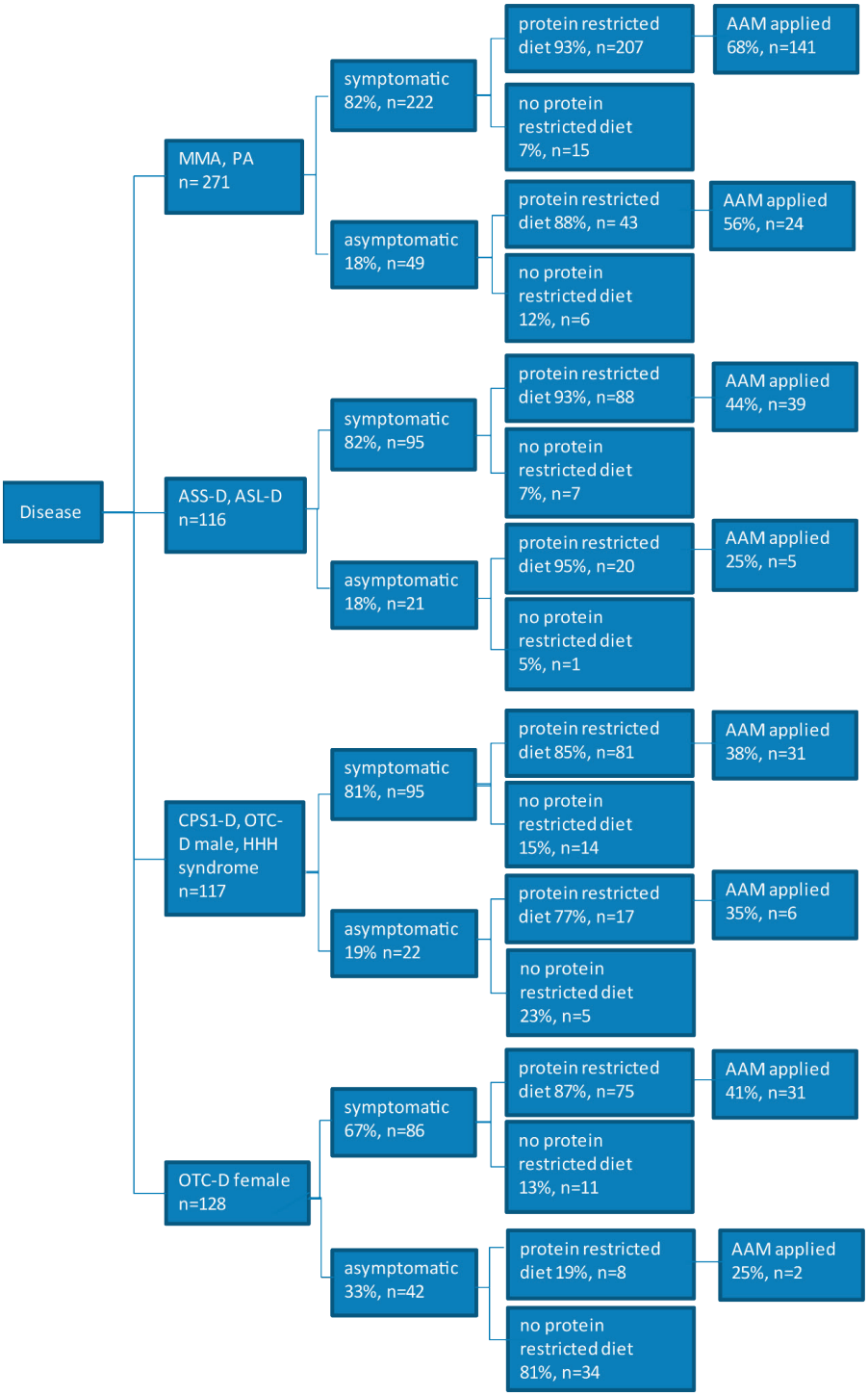
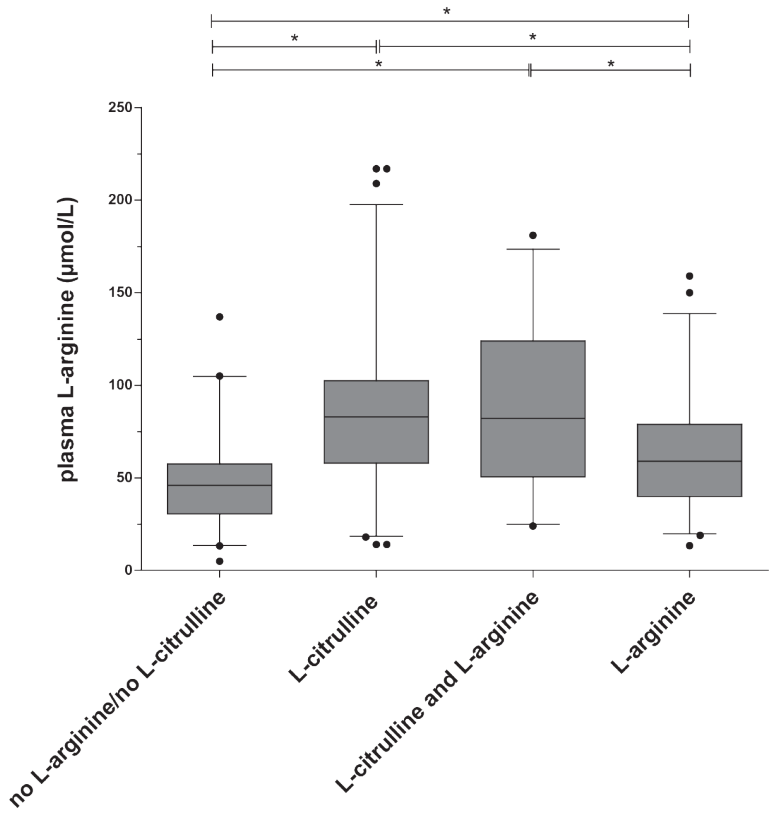
Supplemental tables and figures

Supplementary Figure 1. Dietary treatment in OAD and UCD symptomatic and asymptomatic patients.



Supplementary Figure 2. Plasma arginine levels under the various treatment regimens.



Patients receiving either no L-arginine or no L-citrulline; patients receiving selective L-citrulline; patients receiving both L-arginine and L-citrulline; and patients receiving selective L-arginine. Circles: outliers; whiskers: 5-95 percentile; horizontal line: median; * $p<0.05$

Supplementary table 1. Overview of the included patients.

Disease group	Disease name	Subtype	Patients (n=)	Age at visit (years) (mean ±SD)	Symptomatic (%)	BCAA plasma levels measured (n=)
OAD	MMA	Mut -	15	10.3 ± 8.2	67	8
		Mut 0	87	7.5 ± 6.2	84	57
		CblA	29	11.6 ± 9.8	66	19
		CblB	13	13.5 ± 12.9	92	7
	Total MMA		144	9.1 ± 8.2	79	91
	PA	Alpha	30	8.8 ± 7.5	83	20
		Beta	42	8.0 ± 6.7	81	35
		Not yet classified	55	11.6 ± 8.4	89	34
	Total PA		127	9.8 ± 7.8	85	89
	Total (OAD)		271	9.3 ± 7.9	82	180
UCD	CPS1-D		18	10.5 ± 7.3	94	15
	OTC-D (m)		86	12.9 ± 11.3	77	69
	OTC-D (f)		128	19.4 ± 14.4	67	93
	ASS-D		66	8.5 ± 7.2	83	51
	ASL-D		50	9.8 ± 8.0	80	39
	HHH syndrome		13	15.3 ± 13.8	92	10
	Total (UCD)		361	11.9 ± 10.8	77	277
OAD + UCD	Total		632	11.8 ± 10.9	79	457

Abbreviations. m= male, f= female.

Supplementary table 2a . Countries and included patients.

Country	MMA, PA (n=)	OTC-D male, CPS1, HHH (n=)	ASS, ASL (n=)	OTC-D female (n=)	Total (n=)
Germany	53	16	20	23	112
France	65	31	22	23	141
UK	5	5	5	8	23
Italy	17	12	19	10	58
Netherlands	7	5	5	1	18
Croatia	11	2	1	1	15
Portugal	2	6	3	17	28
Denmark	13	3	5	4	25
Poland	1	3	3	3	10
Switzerland	9	1	5	3	16
Serbia	4	1	2	2	9
Belgium	11	3	2	2	16
Austria	13	2	0	2	17
Czech Republic	10	6	2	5	23
Taiwan	8	4	0	3	15
Greece	2	2	1	0	5
Spain	25	15	18	23	81
Romania	2	0	0	0	2
India	0	0	5	0	5
USA	12	0	0	0	12
Japan	1	0	0	0	1
Total	271	117	116	128	632

Supplementary table 2b. MMA, PA. Countries and dietary treatment.

Country	n= (natural, total protein)	Natural protein %RDA (median)	Total protein %RDA (median)	n= (synthetic protein)	Synthetic protein as % of total protein (median)
Germany	43	94	152	37	44
France	65	97	126	32	35
UK	1	42	139	1	70
Italy	15	134	180	11	45
Netherlands	5	139	205	4	33
Croatia	10	101	148	7	46
Portugal	2	76	153	2	50
Denmark	11	142	151	4	52
Poland	1	101	189	1	47
Switzerland	7	136	147	4	34
Serbia	4	139	139	0	
Belgium	11	116	167	9	35
Austria	12	159	205	11	19
Czech Republic	10	145	219	8	37
Taiwan	7	187	187	0	
Greece	2	148	163	1	15
Spain	20	143	232	13	50
Romania	2	196	246	1	41
India	-				
USA	10	136	218	10	37
Japan	1	74	284	1	74
Total	239	112	159	157	40

Supplementary table 2c. UCD. Countries and dietary treatment.

Country	n= (natural, total protein)	Natural protein %RDA (median)	Total protein %RDA (median)	n= (synthetic protein)	Synthetic protein as % of total protein (median)
Germany	38	92	119	25	26
France	67	105	115	12	27
UK	8	78	103	3	75
Italy	39	77	83	9	29
Netherlands	8	113	177	6	39
Croatia	3	103	103	0	
Portugal	9	106	151	9	27
Denmark	10	141	154	2	29
Poland	8	147	147	4	14
Switzerland	6	101	123	3	40
Serbia	5	133	133	0	
Belgium	5	101	134	2	47
Austria	3	61	100	3	33
Czech Republic	13	96	146	12	36
Taiwan	3	129	138	1	48
Greece	3	104	111	2	42
Spain	44	133	136	13	27
Romania	-			0	
India	4	136	205	1	50
USA	-				
Japan	-				
Total	276	106	127	107	32

Supplementary table 2d. UCD. Countries and sodiumphenylbutyrate prescription.

Country	n= (total UCD patients)	% sodiumphenylbutyrate applied (ASS-D, ASL-D subgroup)	% sodiumphenylbutyrate applied (OTC-D male and female, HHH syndrome and CPS1-D subgroup)	% sodiumphenylbutyrate applied (total UCD)
Germany	59	70	54	59
France	76	9	43	33
UK	18	60	23	33
Italy	41	5	32	20
Netherlands	11	40	67	55
Croatia	4	100	67	75
Portugal	26	0	13	12
Denmark	12	80	14	42
Poland	9	0	0	0
Switserland	7	0	50	29
Serbia	5	0	0	0
Belgium	5	-	33	20
Austria	4	-	50	50
Czech Republic	13	0	73	62
Taiwan	7	0	71	71
Greece	3	0	50	33
Spain	56	56	37	43
India	5	0	-	0

Supplementary table 3a. MMA, PA. Associations within different treatment groups and dietary treatment and plasma BCAA levels.

	Natural protein intake	Total protein intake	Synthetic protein	Plasma L-valine	Plasma L-isoleucine	Plasma L-leucine
Those with a protein restricted diet versus those without	NA	NA	NA	Lower (Ws=15121.5 Z=-3.100, p=0.002)	Lower (Ws=15340.5, Z=-2.840, p=0.005)	ND
Symptomatic versus asymptomatic patients	Lower (Ws=22587.5, Z= -3.111, p=0.002)	Lower Ws=22715.5, Z= -2.273, p=0.023)	**ND**	**ND**	ND	ND
Those receiving AAM versus those not receiving AAM	Not different symptomatic, not different asymptomatic	Higher Symptomatic (Ws=3402.0, Z=-6.654, p<0.001) Asymptomatic (Ws=291.0, Z=-2.440, p=0.015)	NA	Lower symptomatic (Ws=5765.0, Z=-4.197, p<0.001) asymptomatic F(16.212)=3.152, p=0.006	Lower symptomatic Ws=6095.0, Z=-3.189, p=0.001 Asymptomatic not different	ND
Those with AAM and SAA versus those with AAM without SAA	Lower (Ws=2655.0, Z=-3.506, p<0.001)	Lower (Ws=2715.0, Z=-3.280, p=0.001)	ND	ND	ND	ND

NA = not applicable. ND = not different

Supplementary table 3b. UCD subgroups. Associations within different treatment groups and dietary treatment and plasma BCAA levels.

	Disease	Natural protein intake	Total protein intake	L-valine	L-isoleucine	L-leucine	L-arginine
Those with a protein restricted diet versus those without	ASS-D, ASL-D	NA	NA	Not different	Not different	Not different	ND
	OTC-D male, CPS1, HHH syndrome	NA	NA	Not different	Lower (F(16.961)=5.037, p<0.001)	Lower (F(90)=3.5689, p =0.001)	ND
	OTC-D female	NA	NA	Lower (F(90)=5.041, p<0.001)	Lower (F(90)=4.138, p<0.001)	Lower (F(90)=5.015, p<0.001)	ND
Symptomatic versus asymptomatic patients	ASS-D, ASL-D	Lower (Ws=4158.0, Z=-2.832, p=0.005)	Not different	Not different	Not different	Not different	Higher (Ws=712.0, Z=-1.991, p=0.046)
	OTC-D male, CPS1, HHH	Not different	Not different	Not different	Not different	Not different	Not different
	OTC-D female	Not different	Not different	Lower (F(90)=4.861, p<0.001,	lower Ws=2840.0, Z=-2.864, p=0.004,	Lower F(90)=4.690, p<0.001)	Higher (Ws=873.5, Z=-2.760, p=0.006)
Patients with AAM versus those without AAM	ASS-D, ASL-D	ND	ND	Not different	Not different	Not different	ND
	OTC-D male, CPS1, HHH	ND	ND	Not different	Not different	Not different	ND
	OTC-D female	ND	ND	Not different	Not different	Not different	ND

Symptomatic patients receiving AAM versus symptomatic without AAM	ASS-D, ASL-D	Lower (Ws=1243.5, Z=-3.339, p=0.001)	Not different	ND	ND	ND	ND
	OTC-D male, CPS1, HHH	Lower (Ws=767.5, Z=-4.666, p<0.001)	Not different	ND	ND	ND	ND
	OTC-D female	Not different	Not different	ND	ND	ND	ND
Patients with L-arginine versus those without L-arginine	ASS-D, ASL-D	Lower %RDA W=5137.0, Z=-2.453, p=0.014)	Not different	ND	ND	ND	ND
Patients with sodium phenylbutyrate versus those without	ASS-D, ASL-D	Not different	Not different	ND	ND	ND	ND
	OTC-D male, CPS1, HHH	Lower %RDA F(92)=- 5.903, p<0.001)	Lower %RDA (Ws=1318.0, Z=-3.305, p=0.001)	ND	ND	ND	ND
	OTC-D female	Not different	Not different	ND	ND	ND	ND

NA = not applicable, ND = not determined

Supplementary table 3c. Linear regression MMA, PA.

In MMA, PA	L-leucine intake derived from AAM	The amount of natural protein intake %RDA	Age at visit	SAA applied (yes, no)	Symptomatic (yes, no)	
L-valine level	(β-coefficient=-0.279, p<0.001)	(β-coefficient=0.222, p=0.009)	Not significant	Not significant	Not significant	(R^2 =0.197, $R^2_{Adjusted}$ =0.169)
L-isoleucine	Not significant	Not significant	Not significant	Not significant	Not significant	(R^2 =0.085, $R^2_{Adjusted}$ =0.066)

Supplementary table 3d. Linear regression UCD.

In total UCD:	Sodium phenylbutyrate treatment	Age at visit	The amount of BCAA (sum L-valine, L-isoleucine and L-leucine) derived from AAM	The intake of natural protein %RDA	SAA applied (yes, no)	Symptomatic (yes, no)	
L-valine	(β-coefficient=-0.203, p=0.003)	(β-coefficient=0.281, p<0.001)	Not significant	Not significant	Not significant	Not significant	(R^2 =0.142, $R^2_{Adjusted}$ =0.130)
L-isoleucine	(β-coefficient =-0.178, p=0.007)	(β-coefficient=0.238, p<0.001)	Not significant	Not significant	Not significant	Not significant	(R^2 =0.093, $R^2_{Adjusted}$ =0.085)
L-leucine	(β-coefficient=-0.268, p<0.001)	(β-coefficient=0.223, p=0.001)	Not significant	Not significant	Not significant	Not significant	(R^2 =0.180, $R^2_{Adjusted}$ =0.169 respectively).

Supplementary table 4. Overview of prescribed AAM supplements in MMA, PA and UCD.

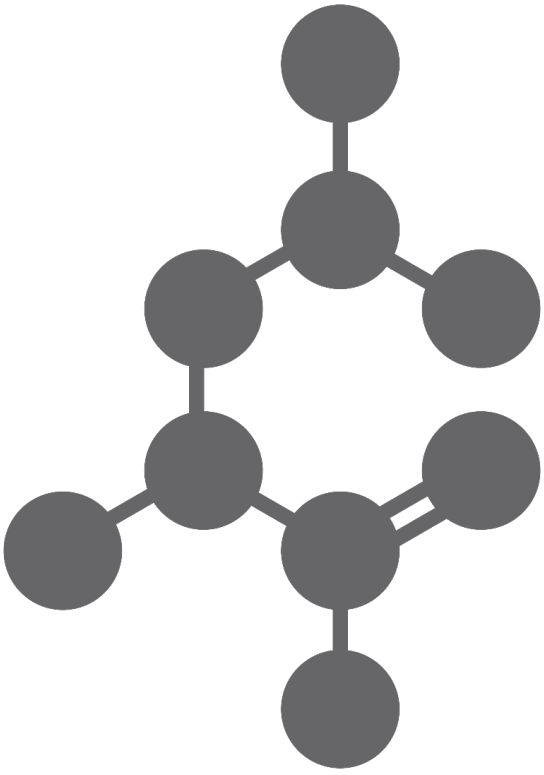
Disease group	n=	Product name	AAM Company	L-valine (mg/100g AAM)	L-isoleucine (mg/100g AAM)	L-leucine (mg/100g AAM)	Total protein intake from AAM (g/kg/d) median ± range
MMA, PA	1	Asadon XMTVI	Nutricia	0.0	0.3	12.8	1.00
	11	OS 2 Secunda	Milupa	0.0	0.0	9.5	0.36 ± 0.38
	2	OS 3 Advanta	Milupa	0.0	0.0	9.5	0.41 ± 0.28
	5	MMA/PA express	Vitaflo	0.0	0.2	9.4	0.29 ± 0.22
	3	IMTV AM1	Nutricia	0.0	0.0	9.3	0.70 ± 0.40
	15	IMTV AM2	SHS	0.0	0.0	9.0	0.48 ± 0.53
	4	IMTV AM3	SHS	0.0	0.0	9.0	0.41 ± 0.02
	2	IMTV AM2	Nutricia	0.0	0.0	9.0	0.67 ± 0.13
	5	OS 2 Prima	Milupa	0.0	0.0	8.1	0.40 ± 0.15
	17	OS 2	Milupa	0.0	0.0	7.6	0.40 ± 0.49
	9	MMA/PA Gel	Vitaflo	0.0	0.2	6.6	0.50 ± 0.40
	11	XMTV 1 Maxamum	SHS	0.0	0.1	6.4	0.40 ± 0.25
	6	OS 1	Milupa	0.0	0.0	5.7	0.50 ± 0.53
	18	XMTV1 Maxamaid	SHS	0.0	0.1	4.1	0.63 ± 0.69
	5	Prompimex 2	Ross	0.0	0.2	2.8	0.55 ± 0.20
	16	MMA/PA Anamix Infant	Nutricia	0.0	0.0	2.1	0.65 ± 0.66
	1	IMTV AM Infant	SHS	0.0	0.1	2.1	1.10
	3	XMTVI	Nutricia	0.0	0.3	2.0	0.60 ± 0.19
	1	XPTM Maxamaid	Nutricia	1.2	1.1	1.9	0.38
	1	MMA/PA cooler	Vitaflo	0.0	0.0	1.8	0.50
	1	Emsogen	Nutricia	0.9	0.9	1.5	0.70
	4	Propimex 1	Ross	0.0	0.1	1.4	0.86 ± 0.47
	6	IMTV	SHS	NA	0.0	NA	0.65 ± 0.30
	1	IMTV AM	SHS	NA	NA	NA	0.80
	1	MT 1	SHS	NA	NA	NA	0.70
	1	Anamix	Nutricia	NA	NA	NA	0.31
	1	IMTV AM	Nutricia	NA	NA	NA	0.22
	1	Maxamaid	Nutricia	NA	NA	NA	2.00
	1	OS pur	Milupa	NA	NA	NA	0.30
UCD	2	E-AM3	SHS	13.0	10.6	17.0	0.20 ± 0.04
	9	E-AM2	Nutricia	13.0	10.6	17.0	0.20 ± 0.15
	2	E-AM1	Nutricia	10.3	8.8	16.3	0.45 ± 0.25
	7	E-AA-mix	Nutricia	14.6	10.4	16.1	0.38 ± 0.19
	28	UCD 2	Milupa	10.7	8.9	15.0	0.25 ± 0.42
	11	UCD 1	Milupa	9.0	7.6	12.8	0.33 ± 0.26
	1	E-AA-mix	Vitaflo	7.2	5.7	9.8	0.35

	23	Dialamine	SHS	4.6	3.3	5.1	0.20 ± 0.51
	2	E-AM Anamix	Nutricia	2.5	2.1	3.4	0.25 ± 0.08
	1	UCD Anamix Infant	Nutricia	2.3	2.3	3.8	0.17
	1	WND1	Mead Johnson	1.2	2.0	2.4	0.20
	1	WND2	Mead Johnson	1.0	1.0	1.9	0.26
	1	UrC-A	Comidamed	1.1	0.9	1.5	1.00
	1	Neocate Advance	Nutricia	0.7	0.6	1.1	1.17
	1	Nutrini Multi Fibres	Nutricia	0.2	0.2	0.3	1.31
	1	Nutrini Peptisorb	Nutricia	0.2	0.2	0.3	0.90
	1	Anamix	Milupa	NA	NA	NA	0.20
	3	UCD	Milupa	NA	NA	NA	0.49 ± 0.20
	1	URC1	Milupa	NA	NA	NA	0.31
	7	E-AM	SHS	NA	NA	NA	0.41 ± 0.17
	1	SHS	SHS	NA	NA	NA	0.30

Ranged based on L-leucine content in AAM products.

Supplementary table 5. Treatment in different MMA subgroups.

MMA total	Mut0	Mut-	CblA	CblB
Total patients	**87**	**15**	**29**	**13**
Symptomatic (%)	84	67	66	92
Protein restricted diet (%)	96	73	79	92
Use of AAM (%)	66	50	28	85
Natural protein intake %RDA (median, range)	115 (42-278)	186 (95-216)	135 (87-301)	135 (53-217)
Total protein intake %RDA (median, range)	162 (57-368)	206 (109-314)	160 (88-333)	171 (99- 381)
Synthetic vs total (median, range)	45 (9-74)	23 (10-50)	32 (26-51)	43 (29-67)
Plasma L-valine (median, range)	81 (22-290)	127 (46-247)	152 (24-237)	66 (38-196)
Plasma L-isoleucine (median, range)	25 (2-115)	46 (23-73)	39 (9-97)	28 (10-67)
Plasma L-leucine (median, range)	70 (26-243)	87 (35-125)	88 (66-196)	44 (30-133)



HOW TO IMPROVE GROWTH IN UCD AND CLASSICAL OAD PATIENTS

Decreased plasma L-arginine levels in organic acidurias (MMA and PA) and decreased plasma branched-chain amino acid levels in urea cycle disorders as a potential cause of growth retardation: options for treatment.

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Abstract

Background and aim: Patients with methylmalonic acidemia (MMA) and propionic acidemia (PA) and urea cycle disorders (UCD), treated with a protein restricted diet, are prone to growth failure. To obtain optimal growth and thereby efficacious protein incorporation, a diet containing the essential and functional amino acids for growth is necessary. Optimal growth will result in improved protein tolerance and possibly a decrease in the number of decompensations. It thus needs to be determined if amino acid deficiencies are associated with the growth retardation in these patient groups. We studied the correlations between plasma L-arginine levels, plasma branched chain amino acids (BCAA: L-isoleucine, L-leucine and L-valine) levels (amino acids known to influence growth), and height in MMA/PA and UCD patients.

Methods: We analyzed data from longitudinal visits made in stable metabolic periods by patients registered at the European Registry and Network for Intoxication Type Metabolic Diseases (E-IMD, Chafea no. 2010 12 01).

Results: In total, 263 MMA/PA and 311 UCD patients were included, all aged below 18 years of age. In patients with MMA and PA, height z-score was positively associated with patients' natural-protein-to-energy prescription ratio and their plasma L-valine and L-arginine levels, while negatively associated with the amount of synthetic protein prescription and their age at visit. In all UCDs combined, height z-score was positively associated with the natural-protein-to-energy prescription ratio. In those with carbamylphosphate synthetase 1 deficiency (CPS1-D), those with male ornithine transcarbamylase deficiency (OTC-D), and those in the hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome subgroup, height z-score was positively associated with patients' plasma L-leucine levels. In those with argininosuccinate synthetase deficiency (ASS-D) and argininosuccinate lyase deficiency (ASL-D), height was positively associated with patients' plasma L-valine levels.

Conclusion: Plasma L-arginine and L-valine levels in MMA/PA patients and plasma L-leucine and L-valine levels in UCD patients, as well as the protein-to-energy prescription ratio in both groups were positively associated with height. Optimization of these plasma amino acid levels is essential to support normal growth and increase protein tolerance in these disorders. Consequently this could improve the protein-to-energy intake ratio.

1| Introduction

Failure to thrive with respect to height in patients with organic acidurias (OAD) and urea cycle disorders (UCD) has been well described [1-6]. Environmental and genetic factors, nutritional factors (essential and functional amino acids), and endocrine factors [7] influence children's growth. Appropriate protein incorporation and protein tolerance is essential for normal growth in children [8, 9]. Dietary protein intake stimulates growth through the production of insulin-like growth factor 1 [10, 11]. Additionally, the dietary protein itself regulates protein turnover via mammalian target of rapamycin (mTOR) signaling by amino acids in skeletal muscle, especially (but not exclusively) by L-leucine and L-arginine [12-14]. One of the most likely underlying cause of the growth retardation in OAD and UCD patients is an installed protein restricted diet [15, 16], with a compensatory high energy intake in order to promote anabolism. Protein and energy requirements are interdependent: as Evans et al. showed recently [17]. In OAD and UCD patients, optimal growth is essential to obtaining sufficient protein incorporation and protein tolerance. In addition, a higher protein tolerance is likely to be associated with less frequent decompensations.

Sufficient intake of essential and functional amino acids is essential to stimulate growth [7]. The most important essential amino acids involved in growth are L-leucine, L-isoleucine, L-valine, L-histidine and L-lysine [18]. Insufficiency of these essential amino acids is associated with growth failure in children [19]. Functional amino acids are defined as "those amino acids that regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of organisms"; they include L-arginine, L-glutamine, L-proline, glycine and L-taurine [20]. L-arginine is a well-known stimulator of the somatotrophic axis that supports physiological growth by inducing growth hormone excretion [21-29]. As L-arginine was shown in a group of ornithine transcarbamylase deficiency (OTC-D) males to improve physiological growth, it may be a therapeutic agent [22]. As indicated previously, branched-chain amino acids (BCAAs) are essential for normal growth, and levels are known to be decreased in MMA/PA and UCD patients alike [15, 16, 30-32].

As functional amino acids (such as L-arginine) and BCAAs play important roles in growth, we aimed to establish any correlations between plasma L-arginine levels, plasma BCAAs levels and height in MMA/PA and UCD patients. To do so, we analyzed data on plasma BCAAs and

L-arginine levels during longitudinal visits in stable disease period of MMA/PA and UCD patients registered in the European Registry and Network for Intoxication Type Metabolic Diseases (E-IMD).

2 | Methods

2.1 | Patient registry and inclusion/exclusion criteria

The E-IMD (URLs: http://www.e-imd.org (website); https://www.eimd-registry.org (registry)) is a European network project that was initiated in 2011 and now includes a web-based patient registry containing comprehensive follow-up data on more than 1,200 individuals with OAD and UCD. A detailed overview of the E-IMD has been published previously (Kölker et al 2015). The data in the E-IMD registry are entered by clinicians and the dietary information in this registry is the diet prescribed by these clinicians/dieticians. Plasma amino acids levels are determined by various methods in the different centers.

The study was approved by the local ethics committee at the coordinating center (University Hospital Heidelberg) and then was approved by all clinical partners. The current publication project was evaluated by the scientific board and approved by the executive board of the E-IMD. In this longitudinal study, we included all first visits registered in the E-IMD registry and all (other) follow-up visits, during stable metabolic periods. We included methylmalonic aciduria (mut-, mut0, cblA and cblB) (MMA; OMIM #251000, CblA OMIM #251100, CblB #251110) and propionic aciduria (PA; OMIM #606054), carbamylphosphate synthetase 1 deficiency (CPS1-D; EC 6.3.4.16; OMIM #237300), ornithine transcarbamylase deficiency (males and females) (OTC-D; EC 2.1.3.3; OMIM #311250), hyperammonemia-hyperornithinemia-homocitrullinuria (HHH; OMIM #238970) syndrome, argininosuccinate synthetase deficiency (ASS-D; EC 6.3.4.5; OMIM #215700) and argininosuccinate lyase deficiency (ASL-D; EC 4.3.2.1; OMIM #207900) patients. We excluded patients with other inherited metabolic diseases, those with an unconfirmed suspicion of an OAD or UCD, those with MMA CblC and CblD subtypes and unclassified type. Other exclusion criteria were kidney and/or liver transplantation, treatment with growth hormone (n=6) and age over 18

years. Clinically symptomatic patients were defined as presenting with a metabolic crisis or long-term complications.

2.2 | Data analysis

Protein prescription was compared with the recommended daily allowance (RDA) as defined in the Protein and amino acid requirements in human nutrition (WHO Technical Report Series 935, 2007), and caloric prescription was compared with the RDA as defined in The National Research Council Washington DC, 2005 [33]. Total protein prescription was calculated by adding the prescription of natural and synthetic protein equivalents in the form of specialized amino acid mixtures and single amino acids (L-isoleucine, L-valine and/or L-leucine) (SAA).

Standard deviation scores (SDS) of height were calculated according to the LMS method [34], which provides a way of obtaining normalized growth centiles. The calculation of SDS for height was based on published reference data [35, 36]. We used the reference data of Cole et al, as they provide complete data for the whole age range examined. The reference data seemed applicable to our study cohort, as reference data were obtained from the UK, and most of the patients in our study are Caucasian Europeans. Plasma amino acid levels were compared with the amino acid reference values provided in table 2.1.5 of the Laboratory Guide to the Methods in Biochemical Genetics by Duran et al [37]. The protein-to-energy ratio (P:E ratio) is calculated on the basis of the amount of protein prescription (grams) per prescription of 100 kilocalories (kcal) per day. The values for natural P:E ratio were compared with values associated with optimal growth described by Evans et al, i.e., a P:E ratio ranging between 1.5 and 2.9 grams protein/100 kcal per day.

On the basis of their metabolic amino acid disturbances and long-term treatment, patients with the following disorders were combined for analysis in four subgroups. The first subgroup consisted of patients with MMA/PA. These OADs are caused by defective enzyme activity in the breakdown in L-valine and L-isoleucine. These patients are generally treated with a protein-restricted diet supplemented either or not in combination with additional amino acid mixtures (AAM) (which lack L-isoleucine and L-valine (AAM-OAD)) and/or SAA.

The second subgroup consisted of patients with CPS-D, male OTC-D and HHH-syndrome and the third subgroup consisted of female patients with OTC-D, which were analyzed separately. Patients within both of these two subgroups are generally treated with a protein-restricted diet supplemented with L-citrulline and/or L-arginine and when necessary with the essential amino acid mixtures for urea cycle disorders (contain essential amino acids) (AAM-UCD). The fourth subgroup consisted of patients with ASS-D and ASL-D. Both disorders show high plasma L-citrulline levels, and are generally treated with a protein- restriction supplemented with L-arginine and when necessary with AAM-UCD.

2.3 | Statistical analysis

SPSS (IBM SPSS Statistics 24.0, IBM Corp., Armonk, NY, USA) was used for descriptive statistics (percentages, mean, standard deviation, median and range). Normality was examined using the Kolmogorov-Smirnov test and quantile-quantile (Q-Q) plots. Student's t-test was performed to compare means if distribution was Gaussian, and the Wilcoxon rank sum test (Ws) was performed if distribution was non-normal. Pearson's correlation coefficient was used to evaluate the correlation between two Gaussian-distributed continuous variables, and Kendall's tau rank correlation was used in the event of non-normal distributions. For the outcomes that were measured longitudinally, we used linear mixed effects models (multilevel analysis) to account for the correlations in the repeated measurements per patient. In particular, we used these models first to study the longitudinal evolutions of the height z-score, and then to investigate the effect of dietary treatment and of plasma amino acid levels on normal growth.

In our analysis of the effect of dietary treatment on height z-scores in MMA, PA the four fixed effects were 1) the natural P:E ratio; 2) prescription of synthetic protein as % RDA; 3) age at visit and 4) clinical symptomatic or not. In our analysis of the effect of dietary treatment on height z-scores in the UCD subgroups two other effects were added, namely whether L-arginine/L-citrulline therapy was applied or not and whether sodium phenylbutyrate was applied or not. We also analyzed interactions in the ratio of fixed effects (age vs. prescription of natural-protein (g/kg/d) to total energy prescription (kcal/d), and age vs. synthetic protein as % RDA).

In our analysis of the effect of plasma amino acid levels on height z-scores in the MMA/PA patients, the six fixed effects were 1) age at visit, and levels of plasma 2) L-arginine, 3) L-lysine, 4) L-leucine, 5) L-isoleucine and 6) L-valine. In our analysis of the effect of plasma amino acid levels on height z-scores in the UCD subgroups, the eight fixed effects were 1) age at visit, and levels of plasma 2) L-arginine, 3) L-lysine, 4) L-ornithine, 5) L-citrulline and plasma 6) L-leucine, 7) L-isoleucine and 8) L-valine. We also analyzed interactions in the fixed effects: age vs. plasma amino acid levels; L-arginine vs. L-lysine levels; L- isoleucine vs. L-leucine levels; L-isoleucine vs. L-valine levels; and L-leucine vs. L-valine levels.

For the random-effects structure we used a build-up approach, starting from random intercepts, and including random slopes with age and higher-order terms. The appropriate random-effects structure was chosen using the Akaike Information Criterion. SPSS (IBM SPSS Statistics 24.0, IBM Corp., Armonk, NY, USA) was used.

3 | Results

3.1 | Description of the study population

Two hundred and sixty-three MMA/PA patients and 311 UCD patients had been registered in the E-IMD registry between 1 February 2011 and 20 May 2016 (table 1, supplementary table 1). At first visit registered, 196 patients with MMA/PA were symptomatic, as were 177 patients with UCD, excluding female individuals with OTC-D. Fifty-six of the females patients with OTC-D—the only X-chromosomal UCD—were symptomatic. Close to fourteen percent of all MMA/PA and UCD patients combined had been diagnosed by newborn screening, 10% by high-risk family/population screening, 2% by prenatal screening, and the remaining 75% by selective metabolic testing. The majority of patients included were Caucasian (80%); the remainders were Asian (10%), African (3%), of mixed ethnicity (4%), and not stated (3%). Most patients were from Europe (95%), with the remainder from Taiwan (2%), the USA (2%), India (0.9%) and Japan (0.1%). In a cumulative number of follow-up visits of 969, a total of 341 patients had been followed-up (161 MMA/PA patients and 180 UCD patients). Per patient, the mean follow-up time was 1.83 years (SD: 1.19). Median time from diagnosis till first visit was 5.10 year, ranging from a follow-up of 0.01 year up to 17.80 years.

3.2 | MMA/PA

3.2.1 | Body length at birth and height z-scores at first visit registered

Birth body-length z-score was close to normal in the MMA/PA patients (median: -0.52, range: -5.53-3.48) (supplementary figure 1). At first visit registered height z-score was below -2SD in 33% of the patients (table 1, supplementary figure 1). The lowest height z-scores were those of MMA patients with the MUT^0 subtype (mean ± SD: -1.70 ±1.56) and cblB subtype (mean ± SD: -1.72 ±1.38) (supplementary table 2), and non-cobalamin responsive patients had lower height z-scores (mean ±SD: -1.69 ±1.59) than the cobalamin responsive patients (mean ±SD: -0.97 ±1.40) ($F(117)=-2.479$, $p=0.015$).

3.2.2 | Dietary treatment and height z-scores

At first visit registered height z-scores did not differ between asymptomatic and symptomatic patients, neither between those with and without a protein restriction. Height z-score was lower in patients receiving AAM-OAD than in those who were not (Ws=13674.5, Z=-2.699, p=0.07). At first visit registered protein restriction (i.e., natural-protein % RDA) was more severe in symptomatic patients than in asymptomatic patients (Ws =17339.5, Z= -2.379, p=0.017) and in patients receiving AAM-OAD than in those who were not receiving AAM-OAD (Ws=14339.0, Z=-2.087, p=0.037). Those patients receiving AAM-OAD had a median natural protein % RDA prescription of 110% RDA, ranging from 18-278% RDA. At first visit registered the mean caloric prescription in all patients was 105% RDA (± SD: 28). At first visit registered the median natural P:E ratio was 1.23, with a range of 0.37-3.33; and the median total P:E ratio was 1.83 (range: 0.63-4.56) (P:E ratio range of >1.5 to < 2.9 gram protein/100 kcal per day is associated with optimal growth [17]). The natural P:E ratio increased with age (rt(199)=0.146, p=0.003). Multilevel analysis showed that height z-score was positively associated with the natural P:E ratio (β-coefficient = 0.305, F(496.865)= 3.419, p=0.001) and negatively associated with the amount of synthetic protein as % RDA (β-coefficient= -0.002, F(451.802)= -3.102, p=0.002) and with patient's age at visit (β-coefficient= -0.052, F(481.130)= -3.466, p=0.001).

3.2.3 | Plasma amino acids: the association with dietary treatment and height z-scores

In MMA/PA patients, plasma L-isoleucine and mainly plasma L-valine were decreased at first visit registered (figure 1A, B), with the lowest levels in MUT^0 and cblB patients (supplementary table 2). Plasma L-arginine levels lay within reference values (figure 1D). At first visit registered patients receiving a protein-restricted diet had lower L-valine levels (F(151)=3.620, p<0.001) than those without protein restriction. Compared to those who were not receiving AAM-OAD, patients receiving AAM-OAD had lower L-valine (Ws=6302.0, Z=-4.415, p<0.001) and L-isoleucine plasma levels (Ws=6697.5, Z=-3.245, p=0.001) at first visit registered. Multilevel analysis showed that height z-score was positively associated with patients' plasma L-valine level (β-coefficient=0.005, t(350.759)=4.157, p<0.001) and L-arginine level (β-coefficient=0.004, t(298.425)=3.203, p=0.002), and negatively associated

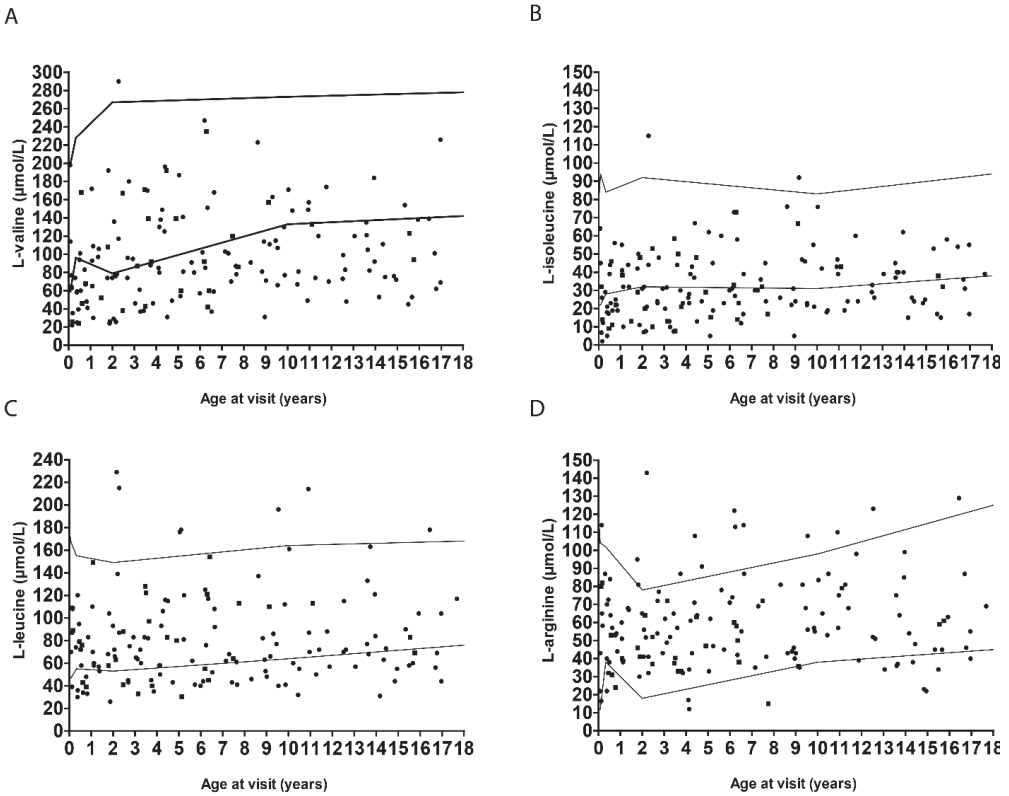
with their age at visit (β-coefficient= -0.033, t(144.698)=-2.064, p=0.041). At first visit registered MMA/PA patients with a height z-score <-2 had a median plasma L-arginine level of 43 µmol/L (range: 12-114) vs. a level of 59 µmol/L (range: 22-143) in those with a height z-score >-2 (Ws=2502.0, Z=-3.546, p<0.001). In those patients with a height z-score <-2, the median plasma L-valine level 76 µmol/L (range: 24-290) at first visit registered vs. a level of 94 µmol/L (range: 22-247) in those with height z-score >-2 (Ws=2996.5, Z=-2.822, p=0.005).

Table 1. Overview of the included patients.

Disease group	Disease name	Subtype	Patients (n=)	Age at baseline (years) (median; range)	Symptomatic (n=/n total)
OAD	MMA	Mut0	86	4.1; 17.7	66/81
		Mut-	12	5.1; 17.7	8/12
		cblA	24	5.2; 15.3	15/23
		cblB	15	6.1; 13.6	11/12
	Total (MMA)		137	4.7; 17.7	100/128
	PA	Alfa	27	5.9; 17.0	21/25
		Beta	39	5.8; 16.4	31/38
		not classified	60	9.3; 17.8	44/50
	Total (PA)		126	6.3; 17.8	96/113
	Total (OAD)		263	5.7; 17.8	196/241
UCD	CPS1-D		15	8.2; 17.2	14/15
	OTC-D (m)		80	7.9; 17.9	57/71
	OTC-D (f)		74	7.2; 17.9	56/69
	ASS-D		77	4.7; 16.8	59/70
	ASL-D		55	7.8; 17.8	39/49
	HHH syndr.		10	4.6; 14.1	8/9
	Total (UCD)		311	7.3; 17.9	233/283
OAD + UCD	Total		574	6.4; 17.9	429/524

Cross-sectional data, one measurement per patient at first visit registered

Figure 1. Amino acid levels in MMA/PA patients.



The lines indicate the upper and lower reference values. Circles indicate symptomatic patients and squares asymptomatic patients.

3.3 | Urea-cycle disorders

3.3.1 | Body length at birth and height z-scores at first visit registered

Birth body-length z-score was close to normal in all UCD patients combined (median: -0.12, range: -4.03-4.49) (supplementary figure 1). Birth length z-score did not differ significantly between OTC-D females and OTC-D males (supplementary table 3). In patients with UCD, height z-scores was lower in patients with cytosolic forms of UCD, i.e., ASS-D, ASL-D patients (mean z-score ± SD: -0.96 ±1.24) at first visit registered than in those with a mitochondrial form of UCD (mean z-score ± SD: -0.39 ± 1.28), i.e., CPS1-D and OTC-D patients, ($F(264)=3.602$, $p<0.001$). At first visit registered body height z-score in those with UCD did not differ between OTC-D females (mean ±SD: -0.36 ± 1.16) and OTC-D males (mean ±SD: -0.20±1.43) (supplementary table 3).

3.3.2 | Dietary treatment and height z-scores

At first visit registered height z-score did not differ between asymptomatic and symptomatic patients, those with and without a protein restriction, or those receiving AAM-UCD and those not receiving AAM-UCD in the following patients: those with CPS1-D, OTC-D males and those with HHH syndrome (subgroup 1); those with ASS-D and ASL-D (subgroup 2); and in OTC-D females (subgroup 3). In subgroup 1 (mean 118 ± SD: 41 vs. mean 149 ± SD: 57 ($F(73)=2.334$, $p=0.022$)) and subgroup 2 (median 100, range: 38-261 vs. median 139, range: 33-261 (Ws 3325, $Z= -2.511$, $p=0.012$)), the natural-protein prescription % RDA was lower in symptomatic patients than in asymptomatic patients at first visit registered. In subgroup 1, the mean energy prescription % RDA was 109.4 (± SD: 28.6); in subgroup 2 it was 109.5 (± SD: 35.5); and in OTC-D females it was 103.5 (± SD: 29.7). At first visit registered the median natural P:E ratio in all UCDs combined was 1.19 (range: 0.47-2.71); and the median total P:E ratio was 1.42 (range: 0.53-3.53) (P:E ratio range of >1.5 to < 2.9 gram protein/100 kcal per day is associated with optimal growth [17]). The natural P:E ratio increased with age ($rt(191)=0.134$, $p=0.006$).

Multilevel analysis in subgroup 2 showed that height z-score was positively associated with the natural P:E ratio (β-coefficient=0.413, $F(159.693)=2.066$, $p=0.040$) and with the use of L-

arginine (β-coefficient=0.624, F(132.042)=2.144, p=0.034). In all UCDs combined, multilevel analysis showed that height z-score was positively associated with the natural P:E ratio (β-coefficient=0.224, F(336.091)=2.100, p=0.036).

3.3.3 | Plasma amino acids: the association with dietary treatment and height z-scores

In the CPS1-D, OTC-D male and HHH-syndrome subgroup, patients' plasma L-leucine levels (F(72)=-3.290, p=0.002) and L-isoleucine levels (F(14.748)=-5.420, p <0.001) were lower in those receiving protein restriction at first visit registered than in those who were not. In OTC-D females, plasma L-valine levels (F(52)= 4.726, p<0.001), L-isoleucine levels (F (53)= 4.700, p<0.001) and L-leucine levels (F(53)= 5.675, p<0.001) were lower in those with a protein-restricted diet than in those without. In all UCD subgroups, patients receiving sodium phenylbutyrate had lower plasma L-valine, L-isoleucine and L-leucine levels at first visit registered than those who were not receiving it, except for L-isoleucine levels in the CPS1-D, OTC-D male and HHH syndrome subgroup (supplementary table 4).

Multilevel analysis in the CPS1-D, OTC-D male, and HHH-syndrome subgroup showed that height z-score was positively associated with patients' plasma L-leucine levels (β-coefficient=0.012, t(84.130)= 3.107, p=0.003). In the CPS1-D, OTC-D male, and HHH-syndrome subgroup patients with a height z-score <-2 at first visit registered had a mean plasma L-leucine level of 62 µmol/L (SD: 37) vs. a level of 93 µmol/L (SD: 36) in those with a height z-score >-2 (F(72)=-2.314, p=0.024).
Multilevel analysis in the ASS-D, ASL-D subgroup showed that height z-score was positively associated with patients' plasma L-valine levels (β-coefficient=0.006, t(144.875)= 2.367, p=0.019). In the ASS-D, ASL-D subgroup patients with a height z-score <-2 at first visit registered had a mean plasma L-valine level of 104 µmol/L (SD: 367) vs. a level of 139 µmol/L (SD: 45) in those with a height z-score >-2 (F(85)=-2.879, p=0.005).

4 | Discussion

Patients with MMA/PA and UCD, treated with a protein restricted diet, are prone to growth failure. To obtain optimal growth and thereby efficacious protein incorporation a diet containing the essential and functional amino acids for growth is necessary. Optimal growth will result in improved protein tolerance and possibly a decrease in the number of decompensations. As essential and functional amino acids are important to growth and amino acid deficiencies are described in MMA/PA and UCD, the purpose of this longitudinal study was to establish how dietary treatment and plasma L-arginine, L-lysine and plasma BCAA levels are associated with height z-scores.

There were four main findings. First, a higher natural P:E ratio was positively associated with height z-score in MMA/PA patients and in all UCD patients combined. Second, height z-scores at first visit registered in MMA/PA as well as in the UCD subgroups did not differ significantly between those with a protein restricted diet and those without. Third, height z-score in MMA/PA patients was positively associated with their plasma L-valine and L-arginine levels. Fourth, while height z-score was positively associated with patients' plasma L-leucine levels in patients in the CPS1-D, OTC-D male and HHH- syndrome subgroup, it was positively associated with patients' plasma L-valine levels in those in the ASS-D, ASL-D subgroup.

The results show that a higher natural P:E ratio is associated with improved height z-score. Although this is in line with a study published by Evans et al [17], we found that the natural P:E ratio in the patients in this study lay below the ratios associated with optimal growth described by Evans et al, implying room for improvement. Natural protein prescription is often close to and/or according to the recommended daily allowances by the World Health Organisations (WHO) [32, 33]. However, the proteins commonly used by MMA/PA and UCD patients are of low biological value and poorly digestible [38]. Whether or not the natural protein prescription could be further increased is currently unknown. It is possible that patients are over-restricted in their natural protein prescription and/or they receive protein of too low biological value. We did not find a significant difference in height z-scores between those with and without a protein restricted diet. Other factors could be important, such as the P:E ratio. Severely affected patients could be prescribed a higher energy diet, in order to compensate for the decreased natural protein prescription and/or protein aversion,

to avoid catabolism and thereby resulting in an inappropriate P:E ratio [38]. The high caloric prescription can also be harmful by causing long-term complications such as obesity and liver steatosis.

As stated above, BCAAs are essential to optimal growth [19, 39]. MMA/PA patients show that a protein-restricted diet was associated with lower plasma levels of BCAA. In MMA/PA patients, we observed a mean plasma L-valine level of 94 µmol/L in those whose height was > -2SD. This suggests that even a small increase in plasma L-valine towards lower levels of normal could help improve growth. MMA/PA patients receiving AAM-UCD had a lower natural-protein prescription and lower plasma BCAA levels than those who were not [32]. Our data also show that height z-score was lower in MMA/PA patients receiving AAM-OAD than in those who were not, underlying the importance of BCAA for optimal growth. Use of AAM-OAD products could also be harmful due to disturbed plasma BCAA ratios [32, 40]. Close monitoring of plasma BCAA and ratio levels and growth and development is extremely important.

In UCD patients our results show that a protein-restricted diet was associated with lower plasma levels of BCAA in the following: in the CPS1-D, OTC-D male and HHH-syndrome subgroup and in OTC-D females. Insufficient protein prescription/intake can cause growth restriction due to low BCAA plasma levels. In UCD patients sodium phenylbutyrate is known to be associated with lower plasma BCAA levels and specific attention and monitoring of plasma BCAA levels [30, 32] and growth in these patient group is highly important. In UCD patients, plasma L-valine and L-leucine levels in low/below normal range were associated with height z-score <-2SD and levels in higher normal ranges with height z-score >-2SD. In UCD patients, AAM-UCD products contain BCAAs and can be beneficial to those with low BCAA levels [32] and growth restriction.

As well as being associated with height z-score, low plasma BCAA levels can cause metabolic decompensations [41], seem essential for optimal functioning of the central nervous system [42-44] and may be important to mitochondrial function in young people [45]—a characteristic that may play a role in disease progression, since mitochondrial dysfunction is a presumable cause of long-term complications in OAD and UCD [46-51].

In view of the metabolic defect, it may not always be possible to optimize MMA/PA patients' plasma L-valine levels. Our data showed that plasma L-arginine levels in MMA/PA patients were associated with height z-scores. To date, there have been no evidence-based recommendations for plasma L-arginine levels in MMA/PA patients, and our current data do not make it easy to give advice on 1) plasma levels that will stimulate growth efficiently and 2) treatment recommendations on L-arginine supplementation in MMA/PA patients. In the study by van Vught, patients who consumed a dose of L-arginine between 2.8 and 3.2 g/day grew faster than those who consumed less than 2.2 g/day [21]. However, the study in question was performed in healthy children, and its data do not provide a reliable basis for recommendations on L-arginine intake in MMA/PA patients. On the basis of the plasma L-arginine levels in those patients with a height z-score < -2SD compared to those with a height z-score > -2SD, we suggest that it could be beneficial to achieve plasma L-arginine levels within high normal range in order to stimulate growth. Second, since the consumption of functional amino acids such as L-arginine is greater than their production during pathologic conditions and growth [52], adequate intake is essential in these circumstances [18, 53].

As well as stimulating the production of growth hormone [21, 22, 54], L-arginine has several other characteristics, such as being a precursor for the production of nitric oxide—an important signaling molecule—, and of creatine [54] and improving anti-oxidant defenses [55, 56]—a characteristic that, in view of the presumed mitochondrial dysfunction in the development of long-term complications, may ameliorate the hazardous progression of the disease [47, 51, 57-59]. Importantly, L-arginine supplementation can have adverse effects [52], such as the induction of hypoglycemia and excessive production of nitric oxide, which can lead to the formation of peroxynitrite [60].

It seems essential to monitor plasma L-arginine levels and verify its possible effect on growth. Further research should then be performed to define recommendations on plasma L-arginine levels and investigate whether increasing natural protein intake is feasible or supplementation of L-arginine is a safe method. Thereafter it is necessary to delineate whether chronic L-arginine supplementation is required or should be restricted to sick-day management. Careful dosing of L-arginine is essential in order to avoid side effects.

Our study and the E-IMD registry had seven main limitations. First, while the E-IMD registry provides an option for mentioning the use of growth hormone therapy, in relatively few patients (n=6) growth hormone was prescribed, which may represent an underestimation of the true number of patients using growth hormone. Second, while the E-IMD registry contains detailed information on dietary prescriptions, it does not describe the patients' real daily intake and consequently there was no information on the quality of the natural protein consumed. Furthermore, between the different centers practice in dietary treatment can vary broadly. Third, the first entry does not reflect the amount of time since diagnosis or dietary treatment and consequently dietary treatment could have been inadequate or different prior to the first assessment. Fourth, the E-IMD registry does not include time between the last meal and the measurement of amino acids. Fifth, due to the high number of participating centers, plasma amino acids levels are determined by various methods, and different reference values are used. There was no documentation or standardization of the process for collection of these amino acids and we did not assess the correctness of measurements and the differences between contributing laboratories. However, most laboratories participate regularly in external quality assessment (ERNDIM). Sixth, due to the use of the E-IMD registry rather than a prospective cohort data are missing. The cross-sectional analysis was done on the available data and is only valid under the missing completely at random assumption. Finally, the follow-up period for some patients was relatively short and furthermore the registry it does not include target height, which would have enabled us to obtain more patient specific height z-scores and the follow-up of patients was relatively short.

Our results are not only important to the MMA/PA and UCD patients but could be of importance for intrauterine growth and for children with other chronic diseases with (resulting) growth restriction. Amino acid deficiencies, and especially arginine, have proven to play an important role in intrauterine growth restriction [61, 62] and plasma L-arginine plays an important role in renal disease and short bowel syndrome [63, 64].

5 | Conclusion

Plasma L-arginine and L-valine levels in MMA/PA patients and plasma L-valine and L-leucine levels in UCD patients were positively associated with height z-scores. To make it possible to formulate recommendations on plasma L-arginine levels, plasma BCAAs levels and optimal natural protein intake (and P:E intake ratio) and to determine whether supplementation of L-arginine and BCAA in MMA/PA and UCD patients could be an important and safe method, further research should be performed.

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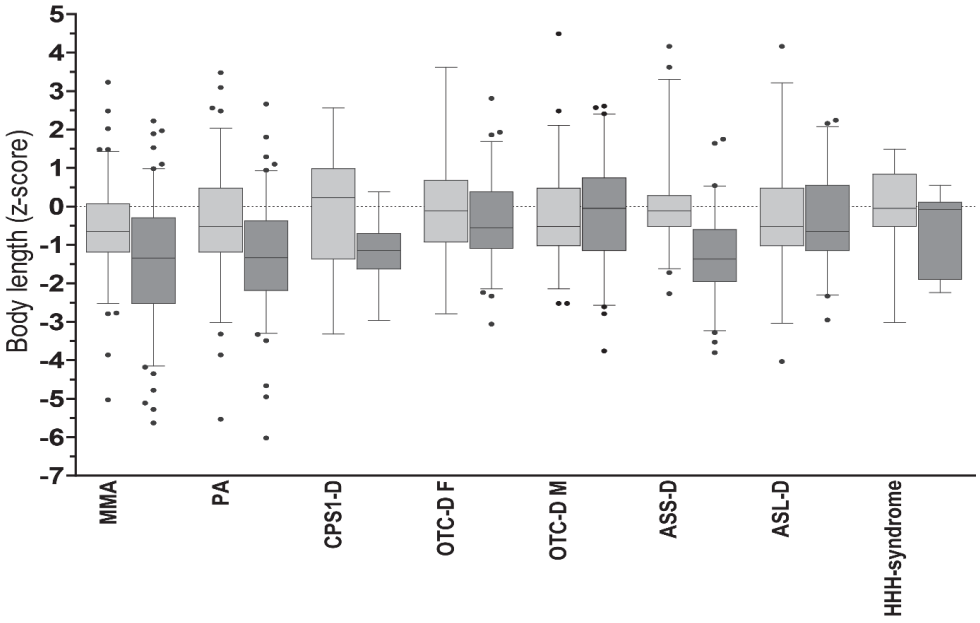
Supplemental tables and figures

Supplementary Figure 1. Body length (z-score) at birth (light grey) vs. height at first visit registered (dark gray) in UCDs and MMA/PA.



Circles, outliers; horizontal line, median; whiskers: 5-95 percentile; * $p<0.05$.

Supplementary table 1. Parameters analyzed in number of patients.

	MMA, PA	ASS-D, ASL-D	OTC-D male, HHH syndrome, CPS1-D	OTC-D female
	n	n	n	n
Patients with a protein restricted diet vs those without	219 vs 14	99 vs 6	80 vs 12	54 vs 14
Symptomatic vs asymptomatic patients	196 vs 45	98 vs 21	79 vs 16	56 vs 13
Patients with AAM vs those without	145 vs 83	39 vs 64	29 vs 62	26 vs 41
Caloric intake	194	94	64	49
Plasma BCAA levels	154	88	75	56
Use of sodium phenylbutyrate vs those without	3 vs 260	34 vs 98	33 vs 72	40 vs 34
Total baseline visits	263	132	105	74
	m	m	m	m
Multilevel analyses	793	311	235	204

Cross-sectional data, one measurement per patient at first visit registered. Number of patients (n) or measurements (m) (longitudinal, total of measurements in multiple patients). Missing data were not included in this table.

Supplementary table 2. Overview of MMA subtypes .

MMA subtypes	Mut0	Mut-	CblA	CblB
Total patients (n=)	**81**	**12**	**23**	**12**
Symptomatic (n=/n total)	66/81	8/12	15/23	11/12
Protein restricted diet (n=/n total)	77/80	9/12	19/21	9/10
Use of AAM (n=/n total)	53/80	6/11	7/21	9/10
Natural protein intake %RDA (median; range)	118; 237	186; 122	132; 213	136; 161
Total protein intake %RDA (median; range)	166; 311	206; 183	155; 214	187; 282
Synthetic vs total (median; range)	40; 73	28; 41	35; 25	41; 19
Plasma L-valine (median; range)	81; 268	139; 201	157; 168	84; 158
Plasma L-isoleucine (median; range)	24; 113	43; 50	39; 53	31; 57
Plasma L-leucine (median; range)	69; 203	87; 90	88; 126	43; 103
Plasma L-arginine (median; range)	52; 131	46; 90	75; 91	40; 72
Z-score height baseline visit (mean ± SD)	-1.7 ± 1.6	-0.9 ± 1.2	-0.7 ± 1.6	-1.7 ± 1.4

Cross-sectional data, one measurement per patient at first visit registered

Supplementary table 3. Overview of the included female versus male OTC-D patients.

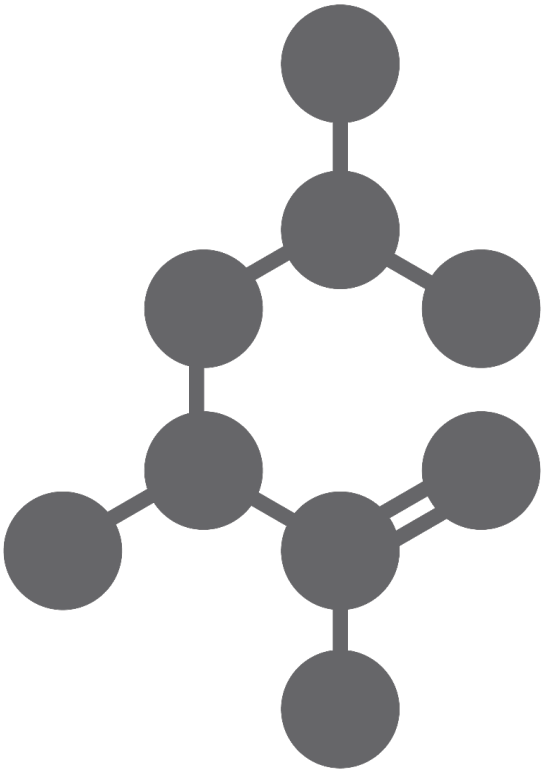
Disease	OTC-D female	OTC-D male	p-value
Age at visit (mean ± SD)	8.01 ± 5.11	7.80 ± 3.65	0.809
Number symptomatic (n=/n total)	56/69	57/71	0.533
Birth length (mean ± SD)	-0.09 ± 1.46	-0.19 ± 1.24	0.691
Body length (mean ± SD)	-0.36 ± 1.16	-0.20 ± 1.43	0.470
Natural protein %RDA (mean ± SD)	121 ± 48	131 ± 48	0.261
Incomplete protein (g/kg/day) (median; range)	0.26; 1.76	0.32; 1.71	0.617
Use of L-citrulline (yes) (n=/n total)	34 /74	33 /80	0.336
Use of L-arginine (yes) (n=/n total)	27 /74	32 /80	0.389
Use of sodium phenylbutyrate (yes) (n=/n total)	40 /74	18 /80	<0.001

Cross-sectional data, one measurement per patient at first visit registered

Supplementary table 4. Comparison of plasma BCAA levels in patients with sodium phenylbutyrate versus those without.

Disease	L-valine	L-isoleucine	L-leucine
ASS-D, ASL-D	mean ± SD: 114 ± 43 mean ± SD: 147 ± 49 F(88)=-2.928, p=0.004	median; range: 31; 56 median; range: 38; 95 Ws=1044.0, Z=-2.100, p=0.036	median; range: 54; 98 median; range: 66; 185 Ws=859.0, Z=-3.305, p=0.001
CPS1-D, OTC-D male, HHH syndrome	mean ± SD: 147 ± 50 mean ± SD: 189 ± 56 F(73)=-3.220, p=0.002	mean ± SD: 42 ± 18 mean ± SD: 52 ± 23 F(74)=-1.926 , p=0.058	mean ± SD: 66 ± 30 mean ± SD: 100 ± 36 F(74)=-4.153, p<0.001
OTC-D female	median; range: 146; 207 median; range: 155; 276 Ws=807.5, Z=-2.002, p=0.045	mean ± SD: 39 ± 14 mean ± SD: 51 ± 22 F(31.540)=-2.189, p=0.036	mean ± SD: 65 ± 22 mean ± SD: 93 ± 38 F(30.812)=-3.319, p=0.004

Cross-sectional data, one measurement per patient at first visit registered



HOW TO MONITOR DISEASE SEVERITY IN MMA AND PA PATIENTS

Fibroblast growth factor 21 as a biomarker for long-term complications in organic acidemias.

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Abstract

Background: There is increasing evidence that long-term complications in organic acidemias are caused by impaired mitochondrial metabolism. Currently, there is no specific biomarker to monitor mitochondrial dysfunction in organic acidemias. Serum fibroblast growth factor 21 (FGF-21) is a biomarker for mitochondrial disease and could be a candidate to monitor mitochondrial function in the deleterious course of disease.

Methods: Data of 17 patients with classical organic acidemias (11 propionic acidemia (PA), 4 methylmalonic acidemia (MMA) and 2 isovaleric acidemia (IVA) patients) were included. The clinical course was evaluated; metabolic decompensations and long-term complications were correlated with plasma FGF-21 levels. Cardiomyopathy, prolonged QT interval, renal failure and optic neuropathy were defined as long-term complications.

Results: Patients ages ranged from 16 months up to 32 years. Serious long-term complications occurred in 8 patients (5 PA and 3 MMA patients). In MMA and PA patients plasma FGF-21 levels during stable metabolic periods were significantly higher in patients with long-term complications (*Mdn* = 2556.0 pg/ml) compared to patients without (*Mdn* = 287.0 pg/ml). A median plasma FGF-21 level above 1500 pg/ml during stable metabolic period, measured before the occurrence of long-term complications, had a positive predictive value of 0.83 and a negative predictive value of 1.00 on long-term complications in MMA and PA patients.

Conclusion: This study demonstrates the potential role of FGF-21 as a biomarker for long-term complications in classical organic acidemias, attributed to mitochondrial dysfunction.

1 | Introduction

The branched chain amino acids isoleucine, valine and leucine are essential nutrients for human growth and development [1]. The enzymes methylmalonyl-CoA mutase, propionyl-CoA carboxylase and isovaleryl-CoA dehydrogenase play pivotal roles in the metabolism of these amino acids. Deficiencies of these enzymes result in three classic organic acidemias, methylmalonic acidemia (MMA; OMIM #251000, #251100, #251110, #277400, #277410), propionic acidemia (PA; OMIM #606054) and isovaleric acidemia (IVA; OMIM #243500). MMA and PA are characterized by the potentially lethal accumulation of toxic metabolites, mainly 2-methylcitric acid, malonic acid and propionyl-CoA [2, 3].

The treatment mainly consists of protein reduction as well as avoidance of catabolism, and survival has greatly improved beyond childhood [4, 5]. However the prognosis is still unsatisfactory with a high rate of severe long-term complications such as renal failure, cardiac arrhythmias, cardiomyopathy, optic nerve atrophy with vision loss, pancreatitis and mental retardation [6-9]. The protein restricted diet may also cause complications, such as failure to thrive [10, 11]. Mitochondrial dysfunction has been established in the pathophysiology of all long-term complications [2, 12-22]. Occurrence of mental retardation is most likely multifactorial, for example cerebral damage during decompensations with metabolic encephalopathy [23], cerebral accumulation of toxic metabolites [24, 25] and presumably mitochondrial dysfunction [12, 13] .

The demonstration of mitochondrial dysfunction is essential to improve the understanding of long-term complications and could be of major importance to develop and potentially evaluate (preventive) treatment strategies by identifying risk groups. The most reliable available evaluation of mitochondrial dysfunction is histological and biochemical assessment of a muscle biopsy. However, muscle biopsies are considered invasive and not acceptable for follow up of mitochondrial dysfunction over the years. Current plasma biomarkers, such as creatine kinase, lactate, pyruvate, alanine and proline levels, and the lactate to pyruvate ratio, have a low sensitivity and specificity [26-28]. Serum fibroblast growth factor 21 (FGF-21) has been suggested as a biomarker for mitochondrial disease. Serum FGF-21 is stable with respect to processing and storage, has a higher specificity and sensitivity compared to other biomarkers and is fast and inexpensive to determine [26, 29-35]. We studied the potential of serum FGF-21 measured in stable metabolic period as a biomarker for long-term complications, attributed to mitochondrial failure, in patients with organic acidemias.

2 | Methods

2.1 | Study design

A total of 17 patients (adults and children) attending the outpatient clinic of the metabolic center of the Erasmus University Medical Center, were selected. Thereafter FGF-21 measurements were done in leftover plasma samples previously collected and stored in the laboratory biobank for research purposes. Of the 17 patients included: 11 were known with PA (case 1-11), 4 with MMA (case 12-15) and 2 with IVA (case 16 and 17). The clinical courses and other laboratory findings were obtained from the medical files. The study was assigned as non-Medical Research Involving Human Subjects Act (WMO) by the Medical Ethical Committee (METC) of the Erasmus University Medical Center. Patients and/or parents of the patients gave permission for publication of their anonymous clinical and laboratory data. Patients and/or parents were informed and aware of the use of left over material for research to which none objected. Cardiomyopathy, prolonged QT interval, renal failure and optic neuropathy were defined as long-term complications. Severe mental retardation and growth failure were not defined as long-term complications. A severe decompensation was defined as a blood pH level below 7.1 and/or ammonia levels above 400 μmol/L [8]. Total protein intake was defined as natural plus synthetic protein intake.

2.2 | Data and statistical analysis

For diagnostic purposes, propionyl-CoA carboxylase activities were measured in fibroblasts at the Section Genetic Metabolic Diseases of the Erasmus University Medical Center, Rotterdam, The Netherlands. The propionyl-CoA carboxylase activity measurement is based upon the incorporation of ^{14}C-labeled CO_2 (given in the form of ^{14}C-labeled sodium carbonate) within the substrate propionyl-CoA. The excess CO_2 that was not incorporated was removed by acidification. At the same time, the incorporation of CO_2 was also measured with pyruvate and with methylcrotonoyl as the substrate. The isovaleryl-CoA dehydrogenase activities were measured in lymphocytes based upon the anaerobic electron transfer flavoprotein fluorescence reduction assay at the Laboratory for Genetic Metabolic Diseases, Academic Medical Center, Amsterdam, The Netherlands. Serum levels of FGF-21 were determined in duplicate using a commercial ELISA kit (Millipore) which is also applied in our

diagnostic process. To establish reference ranges two hundred control subjects were studied, covering all ages, including newborns. Positive controls were 50 patients with a mitochondrial disorder known with an increased plasma FGF-21 level. We established a reference range of 0-200 pg/ml for healthy subjects, which coincides with that of Suomalainen et al [29]. The samples were measured in duplicate and no significant differences between samples (inter-sample variability) were found. Plasma FGF-21 levels up to 3000 pg/ml were linear and measurements were reproducible up to 4000 pg/ml. Higher plasma FGF-21 level than 4000 pg/ml were therefore cut off to a value of 4000 pg/ml. Total protein intake estimated at several time points was based on dietary prescription by a trained metabolic dietitian during standard care, results were compared to the WHO recommended daily allowance (RDA) [36]). Plasma amino acid levels measured during the standard of care were obtained from the medical records to determine nutritional status, and based on reference levels expressed as low (below normal reference level), normal (within reference level) or elevated levels (above reference level). The patients with MMA and PA were selected to assess the association of plasma amino acid levels and plasma FGF-21 levels. Data were summarized by frequencies, median and means with standard deviation (SD). Normal distribution was examined by Kolmogorov-Smirnov tests and quantile-quantile (Q-Q) plots. Student's t-tests were performed to compare means in case of Gaussian and Wilcoxon rank sum tests (Ws) in case of non-normal distributions. Pearson's correlation coefficient was used to evaluate the correlation between two Gaussian distributed continuous variables and Kendall's tau rank correlation (r_τ) in case of non-normal distributions. Paired sample t-tests were performed to compare plasma FGF-21 levels in stable metabolic periods to levels in decompensations within each patient. In analyzing positive predictive value, negative predictive value and odds ratios on the occurrence of long-term complications, we included plasma FGF-21 measurements during stable metabolic periods prior to the development of long-term complications in MMA and PA patients. In defining an optimal cut off point value for FGF-21 we used a receiver operating characteristic (ROC) curve (defining the plasma FGF-21 level with optimal sensitivity and specificity). Since protein restriction may infer FGF-21 increases and since especially leucine and methionine restriction are associated with FGF-21 increase [37], Kendall's tau rank correlation was calculated to test the correlation of total protein intake and methionine or leucine concentrations with plasma FGF-21 levels for each patient individually. Furthermore, we

tested the association of other nutritional parameters, namely valine, isoleucine, alanine, phenylalanine, proline and threonine, with plasma FGF-21 levels. P-values smaller than 0.05 were considered to indicate statistical significances. SPSS data manager (version 24) was used for statistical analyses.

3 | Results

3.1 | Description of the study population

Patient characteristics of all 17 patients with organic acidemia are presented in table 1. Four patients were diagnosed by family or selective newborn screening and one patient was diagnosed prenatally. Eight patients (six with PA and two with MMA) presented in the neonatal period. Two of these patients were initially on mechanical ventilation and one was dialyzed. A total of four patients presented in childhood. These patients presented either with complaints of vomiting, malaise and weight loss; with gastro-esophageal reflux, with encephalopathy or coma.

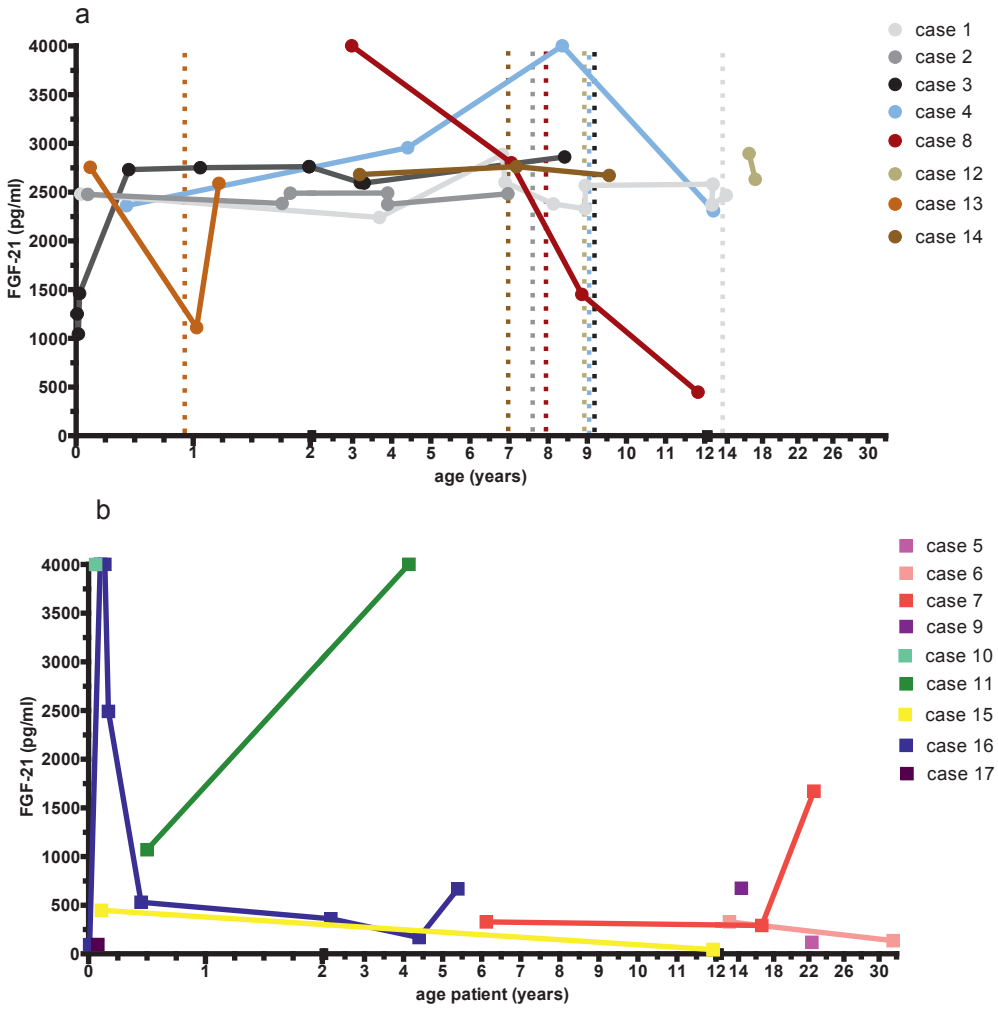
Sixteen patients showed a mild clinical course with no severe decompensations after the initial presentation (table 1). One patient (case 10) died in the neonatal period during the first severe decompensation due to a refractory hemodynamic shock and hyperammonemia. Despite good metabolic control, 8 of the remaining 16 patients developed long-term complications (table 2). The mean age at the first long-term complication was 8.0 years (± SD: 3.4). Five PA patients developed cardiac complications, four of them (case 1, 2, 4 and 8) developed cardiomyopathy between 8 and 13 years of age. In one patient (case 2) this was unexpected as she was diagnosed prenatally by selective screening and she always showed good metabolic control. A rapidly progressive dilated cardiomyopathy lead to cardiac failure and death at eight years of age, four months after the diagnosis of cardiomyopathy. Case 8 had mild cardiomyopathy, but died during a severe metabolic decompensation at the age of twelve years. One patient had prolonged QT interval without signs of cardiomyopathy. Four patients developed renal failure between the age of 11 months and 12 years of age. Two patients developed optic neuropathy, both at the age of 16 years. One of them was also known with reduced left ventricular function, and the other patient was already known with renal failure.

3.2 | Overview of FGF-21 levels and long-term complications

A total of 62 FGF-21 measurements were performed in the 17 patients. Plasma FGF-21 levels were determined before the onset of long-term complications in 7 of the 8 patients with long-term complications (figure 1a). In the remaining patient, FGF-21 measurements were performed in samples obtained after long-term complications had occurred (figure 1a). All FGF-21 levels from samples retrieved before long-term complications developed, were elevated (figure 1a). In two patients (case 1 and 2) plasma FGF-21 levels were already elevated, up to 10 times the upper level of normal, from the neonatal period onwards and remained at the same level with older age. In one patient (case 3) plasma FGF-21 levels increased rapidly from the neonatal period up to eight months of age and remained high thereafter. In another patient (case 8), plasma FGF-21 levels were extremely high before complications and decreased thereafter, as previously mentioned, she eventually died of dilated cardiomyopathy. One patient (case 11) demonstrated high plasma FGF-21 levels during stable disease, at that time point without long-term complications. In an IVA patient without long-term complications (case 16) the highly elevated plasma FGF-21 levels taken during a metabolic decompensation normalized thereafter (figure 1b).

In MMA and PA patients median plasma FGF-21 levels (of each patient) during stable metabolic periods were significantly higher in patients with long-term complications during follow up (median FGF-21= 2556.0 pg/ml) compared to those who did not develop complications (median FGF-21 = 287.0 pg/ml) (Ws=29.00, Z=-2.066, p =0.039) (figure 2a). In MMA and PA patients the individual mean plasma FGF-21 levels during stable metabolic periods (M=2600, SD=843) did not differ from the individual median plasma FGF-21 levels during decompensations (M=2309, SD=619) (t(6)=0.566, p=0.592). None of the patients with a median plasma FGF-21 below 1500 pg/ml during stable metabolic period (n=5) developed long term complications. All, but one patient, developed long-term complications in those with a median plasma FGF-21 above 1500 pg/ml (n=5) (samples taking during stable metabolic period prior to the occurrence of long-term complications) in MMA and PA patients (supplemental figure 1). A median plasma FGF-21 level above 1500 pg/ml during stable metabolic period, measured before the occurrence of long-term complications, has a positive predictive value of 0.83 on long-term complications and a negative predictive value of 1.00 in MMA and PA patients.

FIGURE 1. FGF-21 levels during disease progression.



Dots represent patients with long-term complications (a) and squares those without (b). Colors of the lines define the different patients. Vertical gapped line indicates the occurrence first complication (figure 1a).

Table 1. Patient disease characteristics

Patient	Disease	Mutation	Deduced effect	Enzyme activity	Onset type; age of onset	pH at onset	Lactate level at onset (mmol/L)	NH_3 level at onset (µmol/L)	Lowest pH*	Highest lactate level* (mmol/L)	Highest NH_3 level* (µmol/L)
Case 1	PA	*PCAA* gene Homozygous c.1409T>G	p.(Leu470Arg)	0.03 nmol/h/mg[1]	Neonatal; 2 weeks	7.30	1.7	395	7.21	8.5	132
Case 2	PA	*PCAA* gene Homozygous c.1409T>G	p.(Leu470Arg)	ND	Prenatal diagnosis	7.39	3.3	163	7.22	6.1	255
Case 3	PA	*PCCA* gene, Compound heterozygous c.625G>C/c.923dup	p.(Ala209Pro/ p.Leu308Phefs*23)	ND	Neonatal; 3 days	6.86	1.5	309	7.24	8.3	88
Case 4	PA	ND	NA	0.4 nmol/h/mg[1]	Neonatal; 3 days	7.32	2.0	667	7.35	5.8	134
Case 5	PA	ND	NA	6.6 nmol/h/mg[1]	Childhood; 5 years	7.41	ND	36	7.28	2.1	22
Case 6	PA	ND	NA	4.8 nmol/h/mg[1]	Family screening	7.40	ND	ND	7.17	1.6	90
Case 7	PA	*PCAA* Homozygous C105+2T>G	Splice site	0.20 mmol/h/mg[1]	Early childhood; 7 weeks	7.27	1.9	413	7.28	1.9	167
Case 8	PA	ND	NA	0.12 mmol/h/mg[1]	Neonatal; 2weeks	7.38	NA	42	7.30	5.6	133
Case 9	PA	*PCAA* Heterozygous	p.(Ala209Pro)/p.(Leu30	0.23 mmol/h/m	Neonatal; NA	NA	NA	NA	NA	NA	280

		c.625G>C/c.923 dup	8Phefs*35	g[1]							
Patient	*Disease*	*Mutation*	*Deduced effect*	*Enzyme activity*	*Onset type; age of onset*	*pH at onset*	*Lactate level at onset (mmol/L)*	*NH_3 level at onset (µmol/L)*	*Lowest pH**	*Highest lactate level* (mmol/L)*	*Highest NH_3 level* (µmol/L)*
Case 10	PA	ND	NA	0.26 nmol/h/mg [1]	Neonatal; 10days	7.44	1.2	167	7.15	10.8	1863
Case 11	PA	*PCCB* Homozygous c.1127G>A	p.(Gly376Asp)	ND	Family screening	7.32	2.9	303	7.29	4.7	165
Case 12	MMA	*MMA Mut 0*; Homozygous c.2078delG	p.(Gly693Aspfs*12)	ND	Neonatal; 3 days	ND	ND	172	7.30	3.1	48
Case 13	MMA	*MMAB* gene Homozygous c.556C>T	p.(Arg186Trp)	ND	Early childhood; 6 weeks	7.38	2.3	140	7.31	5.0	66
Case 14	MMA	*MMA Mut 0*; Homozygous c.655A>T	p.(Asn219Tyr)	ND	Neonatal; 1 day	7.01	ND	75	7.24	6.7	106
Case 15	MMA	*MMAA* Homozygous c.433C>T	p.(Arg145)	0.82 nmol/17h/mg [2]	Early childhood; 3 months	7.39	ND	48	7.33	5.8	48
Case 16	IVA	*IVA* Heterozygous c.1241G>A	p.(Arg414Gln)	0.07 nmol/min/mg [3]	Newborn screening	7.34	3.5	49	7.31	5.2	54
Case 17	IVA	*IVA* Homozygous c.716C>T	p.(Thr239Ile)	0.15 nmol/min/mg [3]	Newborn screening	ND	ND	ND	ND	ND	ND

Abbreviations: * reported after initial episode. ND: not determined. NA: not available [1]In fibroblasts, control range: 11-55 nmol/h/mg protein; [2] In fibroblasts, control: 3.5 nmol/17h/mg[2]; [3] In lymphocytes, control range: 0.89-2.13 nmol/min/mg protein. Reference values: pH 7.35-7.45, lactate 0.5-1.7 mmol/L, NH_3 0-50 µmol/L

Table 2. Patient long-term complications

Patient	Disease	Current age	Death	Long-term complications	Cardiac complication; age at diagnosis	Renal failure; age at diagnosis	Optic neuropathy; age at diagnosis	Severe mental retardation; age at diagnosis	Growth failure
Case 1	PA	16 years	-	+	Slight reduced cardiac left ventricular function; 13 years	-	+; 16 years	+; 9 years	+
Case 2	PA	8 years	+	+	Lethal cardiomyopathy; 8 years	-	ND	+; 4 years	-
Case 3	PA	13 years	-	+	Prolonged QT interval; 9 years	-	-	-	-
Case 4	PA	12 years	-	+	Slight cardiac left ventricular hypertrophy; 9 years	-	-	-	+
Case 5	PA	35 years	-	-	-	-	ND	-	-
Case 6	PA	32 years	-	-	-	-	ND	-	-
Case 7	PA	23 years	-	-	-	-	ND	+	-
Case 8	PA	12 years	+	+	Mild cardiomyopathy; 8 years, Left ventricular	+; 12 years	ND	+	+

					dilatation; 12 years				
Patient	*Disease*	*Current age*	*Death*	*Long-term complications*	*Cardiac complication; age at diagnosis*	*Renal failure; age at diagnosis*	*Optic neuropathy; age at diagnosis*	*Severe mental retardation; age at diagnosis*	*Growth failure*
Case 9	PA	15 years	-	-	-	-	-	+	-
Case 10	PA	24 days	+	-	ND	ND	ND	ND	ND
Case 11	PA	4 years	-	-	-	-	ND	+	+
Case 12	MMA	17 years	-	+	-	+; 9 years	+; 16 years	-	+
Case 13	MMA	1 year 4 months	-	+	-	+; 11 months	ND	-	-
Case 14	MMA	18 years	-	+	-	+; 7 years	-	-	+
Case 15	MMA	13 years	-	-	ND	-	ND	-	-
Case 16	IVA	7 years	-	-	ND	-	ND	-	-
Case 17	IVA	9 months	-	-	ND	ND	ND	ND	-

Abbreviations: +: present, -: absent. ND: not determined.

3.3 | Renal function and FGF-21

Plasma creatinine levels did not correlate with plasma FGF-21 levels within the individual patient. Nor did the median plasma FGF-21 level of each patient correlate with the median plasma creatinine level of each patient.

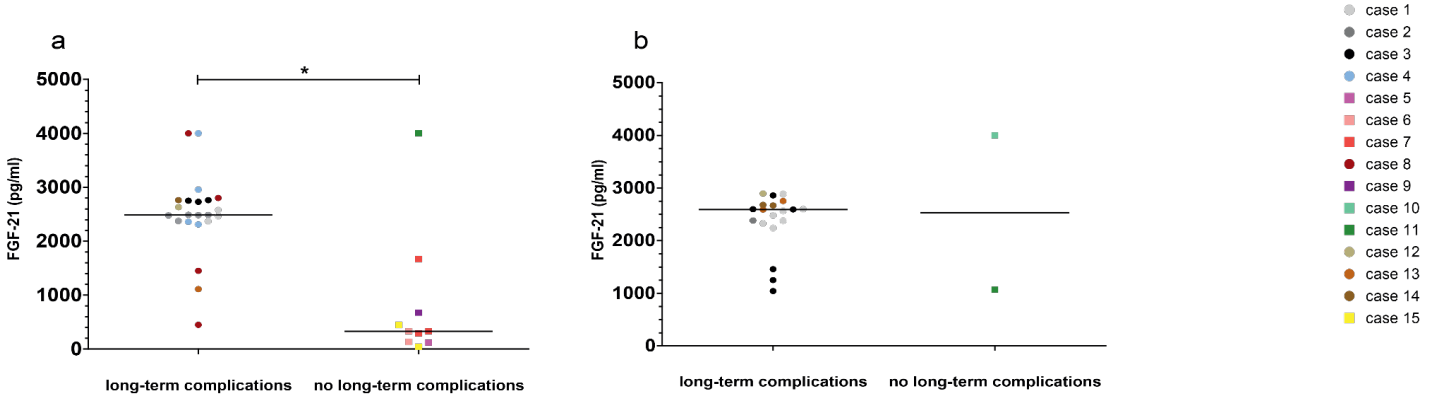
3.4 | Protein restriction and FGF-21

Total protein intake (g/kg/day) did not correlate with plasma FGF-21 levels in any patient nor did median total protein intake (of each patient) correlate with median plasma FGF-21 levels (of each patient). Furthermore, total protein intake as %RDA was not associated with plasma FGF-21 levels, except in one patient (case 3) ($r_\tau(9)=0.957$, $p<0.001$) (supplemental figure 2). In MMA and PA plasma valine levels were low in the majority of the patients (supplemental figure 3). In MMA and PA, plasma FGF-21 levels were not lower in patients with low plasma amino acids levels; valine, isoleucine, leucine, methionine, threonine, alanine, phenylalanine and proline, respectively (supplemental figure 3). Individual plasma amino acid levels were not associated with plasma FGF-21 levels, except for one patient (case 16). In this patient, all three branched chain amino acids (BCAA) were associated with plasma FGF-21 levels (valine ($r(8)=-0.643$, $p=0.026$), isoleucine ($r(8)=-0.618$, $p=0.034$), leucine ($r(8)=-0.618$, $p=0.034$)).

3.5 | Age, height and FGF-21

There is no clear association of plasma FGF-21 levels and age of the patient, in patients with long-term complications (data not shown) as well as in patients without long-term complications (figure 1b). In 16 of 17 patients growth charts were available. Six out of these sixteen patients had moderate to severe growth retardation (table 2). In all of the patients height z-score did not correlate with plasma FGF-21 levels. The median plasma FGF-21 levels did not differ significantly in patients with growth retardation (z-score <-2SD) compared to patients without growth retardation (data not shown).

FIGURE 2. FGF-21 levels and long-term complications.



Measurements during stable metabolic periods, median level indicated by horizontal line (a) and during decompensations, median level indicated by horizontal line (b). Dots represent patients with long-term complications and squares those without. Colours indicate different patients. * significant p-value.

4 | Discussion

This study demonstrates that FGF-21 can be a biomarker for long-term complications, attributed to mitochondrial dysfunction, in classic organic acidemia patients. Plasma FGF-21 levels were high in stable metabolic periods in patients with long-term complications, this was well before the onset of these complications. A median plasma FGF-21 level above 1500 pg/ml during stable metabolic period, measured before the occurrence of long-term complications, had a positive predictive value of 0.83 on long-term complications and a negative predictive value of 1.00 and an odds ratio of 5.0. Plasma FGF-21 levels were not associated with growth, nutritional status or renal function.

Our results give additional proof to the hypothesis that mitochondrial dysfunction plays a role in long-term complications in classical organic acidemia patients [2, 12-21, 38-40]. Plasma FGF-21 levels were high in PA patients before they developed long-term complications, both before severe complications (such as lethal cardiomyopathy) and before milder complications (prolonged QT interval and slight left ventricular hypertrophy). Our observation of high plasma FGF-21 levels in a patient with QT prolongation, suggesting a role of mitochondrial dysfunction, is in line with Baumgartner et al. [41]. In MMA, the renal complications and the high plasma FGF-21 levels could point forward a role of mitochondrial dysfunction in renal failure, which was also proposed by others [15]. Kölker et al. have suggested that mitochondrial dysfunction is not caused by a direct toxicity of methylmalonic acid but by inhibition of the respiratory chain by 2-methylcitric acid, malonic acid and propionyl-CoA, which are increased in MMA as well [3]. Furthermore, plasma FGF-21 levels can be elevated in chronic as well as acute kidney disease and an association between plasma FGF-21 levels and estimated glomerular filtration rate has been found in non OAD patients [42]. In our patient population we did not observe an association between plasma FGF-21 levels and renal function plasma (creatinine levels). Furthermore, plasma FGF-21 levels were already elevated before the occurrence of kidney failure and nor was there an increase in plasma FGF-21 levels when renal function deteriorated.

In stable metabolic periods there is a clear difference in plasma FGF-21 levels in patients with long-term complications compared to those without. Importantly, in this patient cohort the majority of long-term complications occurred between 7 and 9 years of age. This suggests that by determination of plasma FGF-21 levels in stable metabolic period in early childhood

we can potentially identify risk groups for long-term complications. In stable metabolic period only one patient (case 11) had high plasma FGF-21 levels without long-term complications. This patient had his latest visit at the age of 4 years and based on our results and the occurrence of long-term complications mainly between 7 and 9 years of age, this patient can be identified as a potential risk patient for long-term complications. During decompensation, one patient (case 16) had a severe increase in plasma FGF-21 levels, which normalized thereafter. The high plasma FGF-21 levels in this case, could be due to either the decompensation itself or the increased rigorousness of protein restriction; the last was also reflected by the decreased plasma BCAA levels observed during this decompensation. Based on our results, we recommend to measure plasma FGF-21 levels in stable metabolic periods. A median plasma FGF-21 level above 1500 pg/ml measured during stable metabolic period, before the occurrence of long-term complications, seems to predict long-term complications. The negative predictive value of 1.00 observed in this patient cohort should be interpreted with respect to the follow-up time and the fact that long-term complications can occur at later disease stage.

A total of three patients had died, one during the neonatal period (case 10), the others at the age of 8 (case 2) and 12 years (case 8) respectively. Case 8 had initially highly elevated plasma FGF-21 levels in stable conditions, which decreased over the years until the patient died due to lethal cardiomyopathy. This decrease in plasma FGF-21 plasma levels might be the consequence of the potentially lethal mitochondrial dysfunction.

In none of the MMA and PA patients, an association between plasma FGF-21 levels and nutritional status, with respect to protein intake and plasma levels of BCAA, alanine, phenylalanine, methionine, threonine and proline, was found.

There are some potential limitations of this study. Firstly, a retrospective study design cannot make causal inferences and therefore a prospective study is required to confirm our observations. Secondly, this retrospective design could lead to a selection bias and the relatively small number of patients studied could lead to low internal validity.

This study provides a first insight into a potential biomarker for long-term complications in organic acidemias. Long-term complications occur despite good metabolic control [16, 43]. We suggest that mitochondrial dysfunction is an ongoing process independent of metabolic

control. It is important to focus on the role of preventive treatment strategies. A potential preventive therapy is anti-oxidants administration. To date, the beneficial efficacy of anti-oxidant therapies varies, ranging from improvement of long-term complications to no beneficial effect [18, 39, 40, 44]. Absence of therapy efficacy can be due to the relative late initiation of anti-oxidants in disease progression. First, the results observed in this study, namely the association between plasma FGF-21 levels and the occurrence of long-term complications as well as the high positive and negative predictive value of a median plasma FGF-21 level above 1500 pg/ml, should be replicated in another cohort of classical organic acidemia patients. Other biomarkers for mitochondrial dysfunction, like growth differentiation factor 15 (GDF-15), should be included in order to identify their role and relation with the occurrence of long-term complications in these disorders and compare it to plasma FGF-21. Once the FGF-21 results are confirmed and role of GDF-15 established, further research should determine if FGF-21 or other mitochondrial markers could play a role in the evaluation of preventive strategies concerning the consequences of mitochondrial impairment, and if FGF-21 plays a pathogenic role in the development of mitochondrial impairment.

5 | Conclusion

This study provides new insights into the potential role of FGF-21 as a biomarker for predicting long-term complications in organic acidemias. Increased plasma FGF-21 levels and long-term complications are attributable to mitochondrial dysfunction. Further research should be performed in another cohort of patients to verify our observations and to investigate the potential role of FGF-21 as a biomarker of (preventive) treatment efficacy.

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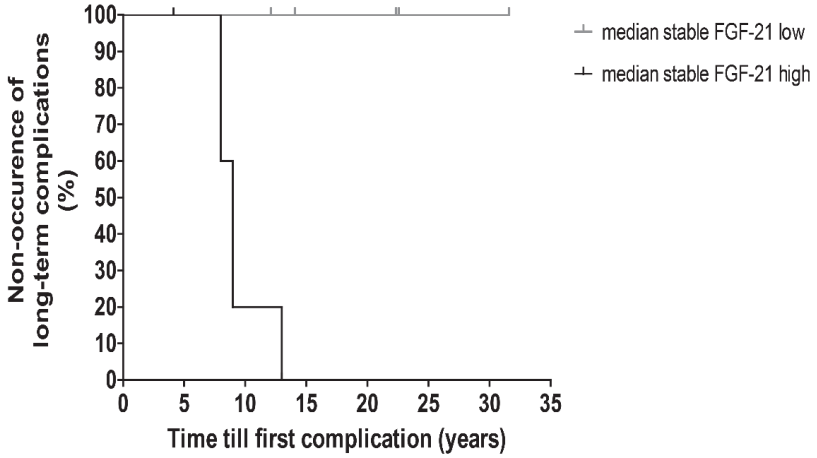
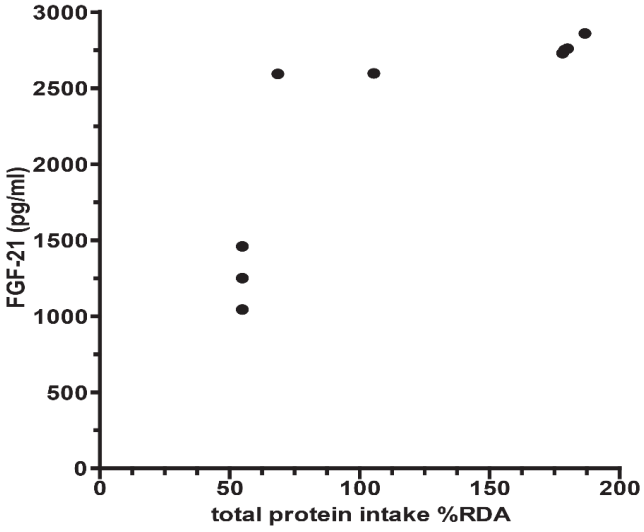
Supplemental tables and figures

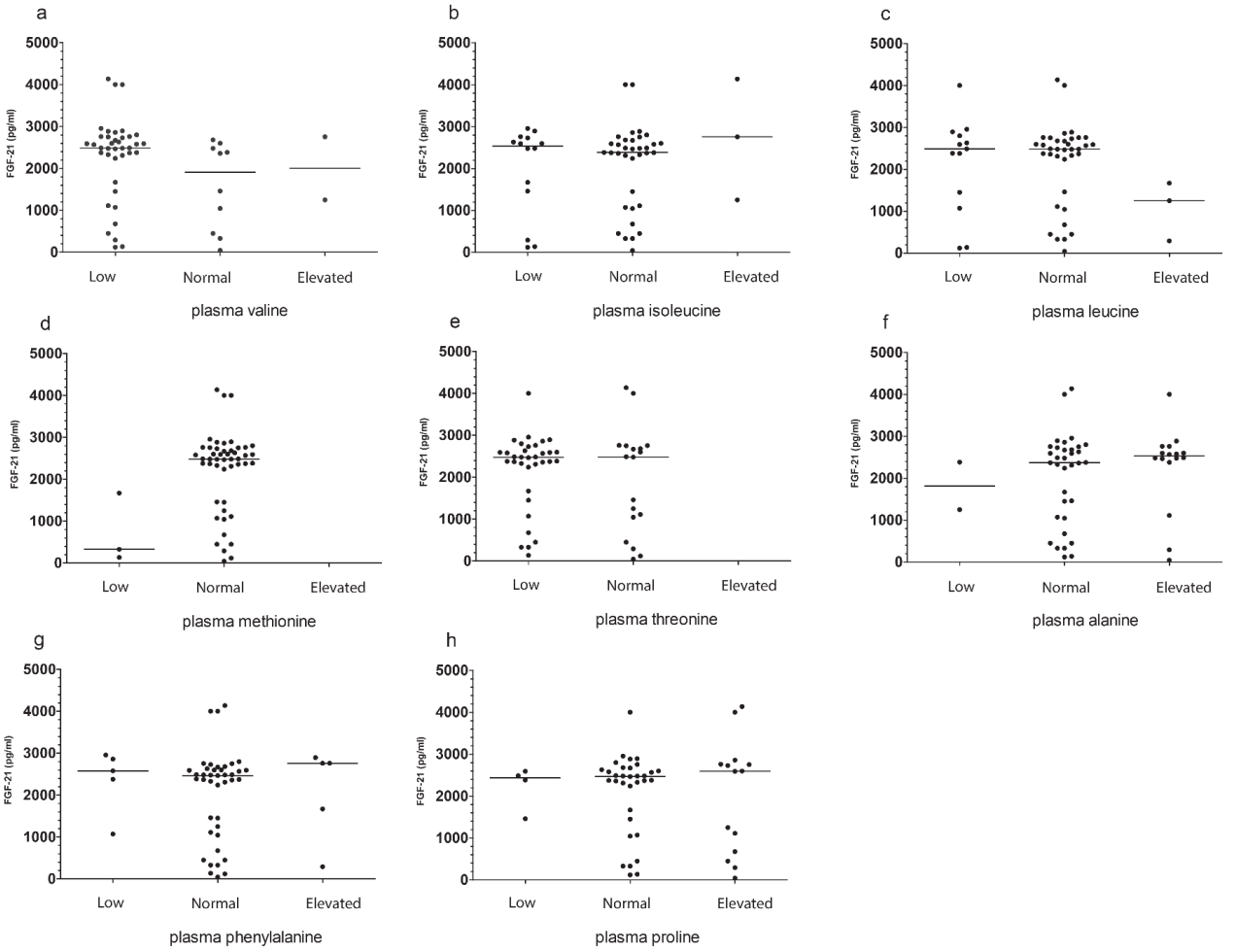
Supplemental figure 1. Survival curve (percentage of non-occurrence of long-term complications).



Percentage at x-axis defines the total percentage of those without long-term complications. The occurrence of long-term complications in patients with a median FGF-21 level above 1500 pg/ml (= high), measured during stable metabolic period before the onset of long-term complications, versus patients with a median FGF-21 below 1500 pg/ml (= low) measured during stable metabolic period.

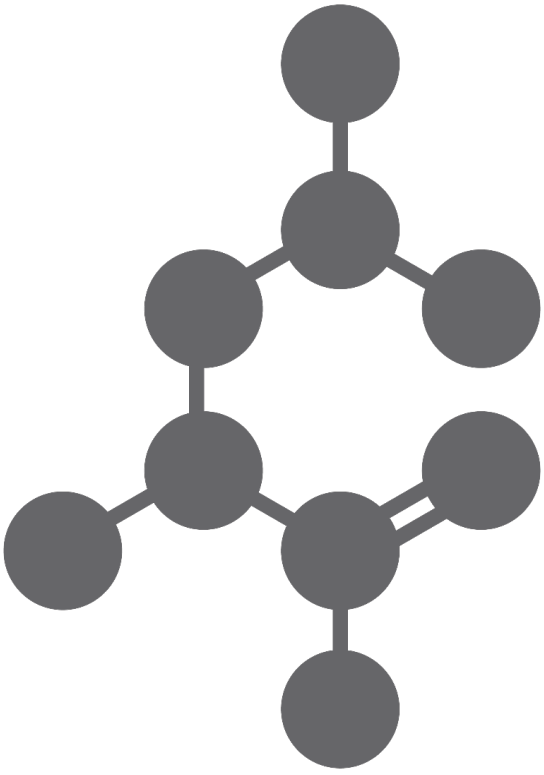
Supplemental figure 2. Plasma FGF-21 levels compared to total protein intake %RDA in case 3.





Supplemental figure 3. Plasma FGF-21 levels compared to low (below lower level of reference values), normal (within reference values) or elevated (above reference values) amino acid plasma levels.

All measurements of each patient included.



NEWBORN SCREENING IN MMA AND PA PATIENTS

Retrospective evaluation of the Dutch pre-newborn screening cohort for propionic acidemia and isolated methylmalonic acidemia: what to aim, expect and evaluate from newborn screening?

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Abstract

Background: Evidence for effectiveness of newborn screening (NBS) for propionic acidemia (PA) and isolated methylmalonic acidemia (MMA) is scarce. Prior to implementation in the Netherlands, we aim to estimate the expected health gain of NBS for PA and MMA.

Methods: In this national retrospective cohort study, the clinical course of 76/83 Dutch PA and MMA patients, diagnosed between January 1979 and July 2019, was evaluated. Five clinical outcome parameters were defined: adverse outcome of the first symptomatic phase, frequency of acute metabolic decompensations (AMD), cognitive function, mitochondrial complications and treatment-related complications. Outcomes of patients identified by family testing were compared with the outcomes of their index siblings.

Results: An adverse outcome due to the first symptomatic phase was recorded in 46% of the clinically diagnosed patients. Outcome of the first symptomatic phase was similar in 5/9 sibling pairs and better in 4/9 pairs. Based on the day of diagnosis of the clinically diagnosed patients and sibling pair analysis, a preliminary estimated reduction of adverse outcome due to the first symptomatic phase from 46% to 36-38% was calculated. Among the sibling pairs, AMD frequency, cognitive function, mitochondrial and treatment-related complications were comparable.

Discussion: These results suggest that the health gain of NBS for PA and MMA in overall outcome may be limited, as only a modest decrease of adverse outcomes due to the first symptomatic phase is expected. With current clinical practice, no reduced AMD frequency, improved cognitive function or reduced frequency of mitochondrial or treatment-related complications can be expected.

1 | Introduction

Propionic acidemia (PA, OMIM #6060054) and isolated methylmalonic acidemia (MMA, OMIM #251000, #251100, #251110, #277400 and #277410) are caused by enzyme and cofactor deficiencies involved in propionyl-CoA breakdown. The clinical course usually starts with an acute metabolic decompensation (AMD) in the neonatal period (early onset, EO) or thereafter (late onset, LO) [1]. The first symptomatic phase frequently causes irreversible neurological damage, manifesting as movement disorders in varying severity and/or psychomotor retardation. This neurological damage is often reflected by white matter lesions and/or basal ganglia hyperintensities on brain imaging. In addition, the clinical course is characterized by frequent AMD, cognitive deficits and long-term complications [2].

A diagnosis before the first symptomatic phase, feasible through newborn screening (NBS), could theoretically prevent the neurological damage [3]. However, the majority of patients is symptomatic well before NBS results can be available, limiting the potential gain [4, 5]. Four studies described the potential additional value of NBS, but each with limited sample sizes and follow-up time [6-9], resulting in controversy about the potential effectiveness of NBS for PA and MMA.

Many efforts have been made to achieve consensus on which diseases are suitable for screening, both on a global level by the World Health Organization (WHO) [10, 11] (Supplementary Notes 1), and on European level [12-14]. Despite these efforts, it is still debatable which diseases to screen for and it is not clear on what criteria decisions should be based, resulting in divergent conclusions. Regarding PA and MMA, NBS implementation has been recommended in the United States of America[15], but PA and MMA were not included in the European list of 26 disorders to be considered for NBS expansion [13]. Nevertheless, several European countries implemented NBS for PA and MMA [5].

In the Netherlands, the Health Council decided based on the expected health gain for LO patients that NBS for PA and MMA met the screening criteria and will start in October 2019[16]. NBS will be performed based on increased C3-carnitine and increased C3/C2 carnitine ratio, complemented with a second-tier test on increased 2-methylcitric acid and methylmalonic acid. As the heel prick is performed within 3-7 days after birth, the aim is to report a positive second-tier test within 11-21 days after birth.

For the addition of new diseases to NBS, three WHO criteria seem of particular importance: clearly defined objectives (WHO#2), evidence for effectiveness (WHO#4) and careful

evaluation (WHO#9) [11] (Supplementary Notes 1). To date, for PA and MMA these three criteria are not sufficiently met. Also, despite statements that good long-term outcome is the ultimate goal and that monitoring is necessary to evaluate NBS programmes, long-term outcome is rarely monitored in a standardized manner [13].

We performed a national retrospective cohort study including 76/83 Dutch PA and MMA patients. We aim to define which objectives could be attained with NBS implementation. Hereto, we estimate the expected health gain in the Netherlands by comparing siblings diagnosed via family testing with their older index sibling. In addition, we explore how evaluation of NBS implementation can be performed by assessing newly defined clinical outcome parameters. Lastly, we use insights obtained from studying this pre-NBS cohort to guide counseling and surveillance of NBS cohorts.

2 | Methods

2.1 | Patient inclusion

In this nationwide retrospective cohort study, patients clinical records from all six Dutch metabolic centers were analyzed. Patients were eligible for inclusion if PA or MMA was confirmed by enzymatic activity analysis or mutation analysis of *PCCA*, *PCCB*, *MUT*, *MMAA*, *MMAB*, *MCEE* or *MMADHC*. Patients or their legal guardians signed an informed consent form, approving analysis of their clinical records and publication of the anonymous data, in agreement with institutional and national legislation. The study was approved by the Ethical Committees of the University Medical Centers Utrecht (17-490/C) and Rotterdam (MEC-2018-1312), on behalf of all Ethical Committees of involved University Medical Centers.

2.2 | Data collection

Data collection was performed by two investigators (HH, FM). Data was extracted from both paper and electronic patient files. Data was collected from the first symptomatic phase until the last follow-up visit. Data collection was focused on patient and family characteristics, outcome of the first symptomatic phase, AMD, hospital admissions, cognitive and psychomotor development and long-term complications. All data was entered in OpenClinica open source software, version 3.12.2.

EO was defined as an episode of severely reduced clinical condition starting in the first 28 days of life, and LO as symptoms starting after 28 days of life [1]. Patients identified through family testing based on an affected older (index) sibling were regarded a separate category.

2.3 | Outcome parameters

To evaluate clinical outcomes, five grouped outcome parameters were defined (Supplementary Notes 2). "Adverse outcome (AO) of the first symptomatic phase" was categorized based on the presence of movement disorders that developed during or right after the first symptomatic phase, objectified during follow-up examinations by pediatric neurologists and physiotherapists shortly after the first symptomatic phase, and/or the presence of basal ganglia hyperintensities or white matter abnormalities detected at the first time of brain imaging after the first symptomatic phase (Supplementary Notes 2). "AMD frequency" was defined based on the frequency of (impending) AMD requiring hospital admission per patient year (PY) in the first four years of life, other than the first symptomatic phase (Supplementary Notes 2). "Cognitive function" was distinguished in three categories based on neuropsychological test results, or in the absence of neuropsychological test results on educational level or professional employment[17] (Supplementary Notes 2). Long-term complications were divided into mitochondrial, treatment-related and miscellaneous complications. Patients were categorized into six categories based on the number of mitochondrial complications present at a certain age. These mitochondrial complications included 21 complications likely to be caused by mitochondrial failure[2] (Supplementary Notes 2). Complications with potential treatment-related etiology included decreased bone mineral density (BMD), growth retardation and obesity[2] (Supplementary Notes 2). Growth retardation was defined present when height to weight was < -2 SD at any moment during follow-up. Other complications, with no evidence for a sole mitochondrial or treatment-related etiology, were regarded miscellaneous complications. These complications included pes planovalgus, port-a-cath infections, teeth enamel defects, urolithiasis and gout [2].

2.4 | Data-analysis

Each outcome parameter was evaluated in the complete cohort, separate for both PA and MMA patients and separate for both EO and LO patients. In addition, for all AMDs age at

decompensation, admission duration, need for intensive care unit (ICU) admission and AMD triggers were evaluated. For each complication assessed, prevalence was calculated.

As a proxy for the potential health gain of NBS, all five clinical outcome parameters were compared between patients identified by family testing and their index siblings. The expected health gain was calculated by extrapolating findings of the sibling comparison on AO of the first symptomatic phase to the entire cohort, if NBS would have been implemented. Calculations were performed for six possible days that definitive NBS results could become available: day 0, 3, 7, 11, 15 and 21 after birth.

Assessment of risk factors for each outcome parameter was performed by comparing disease types, for both PA and MMA presentation types and for MMA vitamin B12 responsiveness.

All statistical analyses were performed in R programming language. Results are expressed as either means or medians with standard deviations and ranges for quantitative data and as number and percentage for qualitative data. Student's t-tests were performed for quantitative data and Fisher's exact tests for qualitative data. P-values were adjusted according to the Bonferroni method when multiple testing was performed, adjusted p-values < 0.05 were considered statistically significant. P-values solely reported in the text for the assessment of risk factors of clinical outcome parameters were calculated using Fisher's exact tests and were not adjusted, as no multiple testing was performed.

3 | Results

3.1 | Patient characteristics

Of all 83 PA and MMA patients in the Netherlands diagnosed between January 1979 and July 2019, 76 patients were included. Five patients refused to participate and clinical records of 2 deceased MMA patients could not be retrieved. For nearly all patients, data was available from the first symptomatic phase to the last moment of follow-up. The cohort included 31 PA patients from 24 families and 45 MMA patients from 40 families. Between 01-01-1998 and 31-12-2017, 46/76 patients (PA: *n* = 19, MMA: *n* = 27) were diagnosed, with a median of 2 patients per birth year (range: 1 – 5). In this period 3.729.128 live births were registered, providing an estimated incidence in the Netherlands of 1:81.000 for PA and MMA combined, 1:196.000 for PA and 1:138.000 for MMA. Presentation type was EO in 29, LO in 34, family testing in 12 and unknown in one patient (Table 1). Clinical data of 5 PA sibling pairs and 1 trio, and 3 MMA sibling pairs (*n* = 9) was available for sibling comparison (Table S1). Two MMA patients identified through family testing could not be compared with their index siblings, because medical records of the index sibling were not available.

Mutation analysis was available in 52/76 patients and revealed 25 previously unreported mutations, including 7 mutations in *PCCA*, 5 in *PCCB*, 8 in *MUT*, 2 in *MMAA* and 3 in *MMAB* (Table S2). Pregnancy and delivery were uncomplicated in the majority of the patients' mothers. Birth weight of MMA patients was significantly lower compared to PA patients, in line with previous reports[1] (Table S3). In most patients, the first symptomatic phase was characterized by lethargy and anorexia. Significantly more LO patients presented with vomiting. Plasma ammonia at presentation was significantly higher in EO patients, resulting in significantly more EO patients requiring dialysis (Table S4).

Table 1. Baseline characteristics of the Dutch cohort of PA and MMA patients.

	Propionic acidemia (*n* = 31)		**Methylmalonic acidemia (*n* = 45)**	
Presentation type	Early onset Late onset Family testing Unknown	*n* = 15; 48% *n* = 8; 26% *n* = 7; 23% *n* = 1; 3%	Early onset Late onset Family testing	*n* = 14; 31% *n* = 26; 56% *n* = 5; 11%
Presentation age	Early onset Late onset Family testing	6.05 ± 6.12 days 14.1 ± 22.9 years 0.07 ± 0.19 years	Early onset Late onset Family testing	2.07 ± 1.77 days 1.41 ± 2.74 years 0.32 ± 0.72 years
Genotype	*PCCA* *PCCB* No mutation analysis	*n* = 8; 26% *n* = 7; 23% *n* = 16; 52%	*MUT* *MMAA* *MMAB* No mutation analysis	*n* = 26; 58% *n* = 8; 18% *n* = 7; 16% *n* = 4; 9%
Enzyme activity	0.0 – 0.4 nmol/h/mg 0.5 – 2.0 nmol/h/mg Not measured	*n* = 10; 32% *n* = 5; 16% *n* = 16; 52%		
Vitamin B12 responsiveness			Responsive Not responsive	*n* = 21; 47% *n* = 24; 53%
Age at last follow-up		19.2 ± 15.1 years		17.3 ± 12.1 years
Mortality		*n* = 7; 23% (4 EO, 1 LO, 2 family)		*n* = 2; 4% (1 LO, 1 family)

EO: early onset; LO: late onset; family: family testing. Early onset: presentation ≤28 days of life; Late onset: presentation >28 days of life. Qualitative data is expressed as number and percentage, quantitative data as mean ± standard deviation.

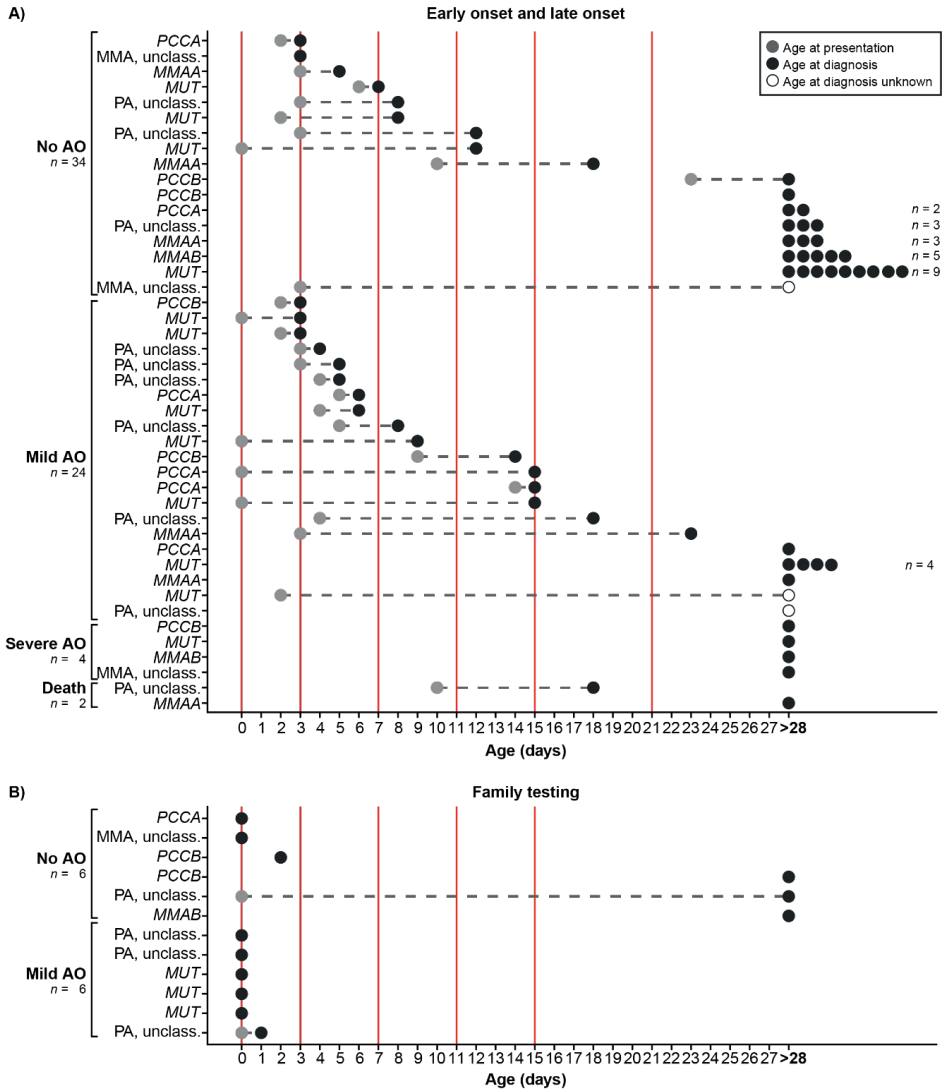
3.2 | Adverse outcome of the first symptomatic phase

An AO of the first symptomatic phase was recorded in 36/76 (47%) of all patients and in 30/64 (47%) of the clinically diagnosed patients (Figure 1). Movement disorders were recorded in 24/36 patients. Basal ganglia hyperintensities were observed in 18, white matter abnormalities in 14 and both in 6 patients. In 19/26 patients in whom white matter abnormalities and/or basal ganglia hyperintensities were observed, brain imaging was performed within 3 days to 6 months after the first symptomatic phase and in 7/26 patients later in life (range: 9 months to 23 years). In 3 of these patients also a movement disorder was recorded, and in all 7 patients the observed abnormalities were considered to be long-existent based on unchanged clinical symptoms and imaging characteristics.

In EO patients an AO due to the first symptomatic phase was either absent or, more often, mild, whereas in LO patients most often no AO due to the first symptomatic phase was recorded. If an AO was recorded in LO patients it was either mild or severe, which is represented by a significant difference in mild AO due to the first symptomatic phase in EO versus LO patients (Table 2, Figure S1). AO due to the first symptomatic phase tended to be less frequent in patients identified through family testing than in their siblings (better outcome of the first symptomatic phase in 4/9 sibling pairs, similar in 5/9) (Table S1). Symptoms or signs present during the first symptomatic phase, nor presentation type in PA or MMA, nor vitamin B12 responsiveness in MMA, was associated with an increased risk for an AO (Table S5).

The expected health gain of NBS for PA and MMA in the Netherlands was estimated based on the moment of presentation of all EO and LO patients in this cohort and based on analysis of sibling pairs (Figure 1, Table S1, Table 3). Theoretically, the earlier NBS results are available, the more patients could be protected from an AO due to the first symptomatic phase (Figure 1, Table 3). In this cohort, NBS results available at day 0 of life could have prevented an AO in 14 patients, reducing the percentage of an AO during the first symptomatic phase from 46% to 23%. NBS results available between day 11 and 21 could have prevented an AO in 6 or 5 patients, reducing the percentage from 46% to 36-38% (Table 3).

FIGURE 1. Age at onset and age at diagnosis for PA and MMA patients.



The y-axis depicts the patients, ordered on type of adverse outcome and age at diagnosis. The x-axis depicts the patient age in days, with a maximum of 28 days (early onset). Light gray circles depict age at presentation, dark gray circles depict age at diagnosis, open circles depict in which patients the exact age at diagnosis is unknown. For all ages at presentation and ages at diagnosis $\geq$ 28 days of age (late onset), the circles have been placed at 28 days. The vertical red solid lines depict the potential moments NBS results could become available, in line with Table 3. On the left the severity of adverse outcomes (AO) due to the first symptomatic phase are listed: no adverse outcome, mild, severe or death due to the first symptomatic phase.

Table 2. Grouped outcome parameters according to presentation type.

	Early onset (*n* = 29)	Late onset (*n* = 34)	P-value	Bonferroni correction
Adverse outcome of first sympt. phase				
No adverse outcome	*n* = 11; 38%	*n* = 23; 64%	0.024	NS
Mild adverse outcome	*n* = 17; 59%	*n* = 6; 21%	0.001	**0.006**
Severe adverse outcome	*n* = 0; 0%	*n* = 4; 12%	0.118	NS
Death due to first presentation	*n* = 1; 3%	*n* = 1; 3%	1.000	NS
AMD frequency				
None: no AMD in first four years	*n* = 3; 10%	*n* = 13; 38%	0.019	NS
Mild: >0.0 ≤ 0.5 AMD/PY	*n* = 1; 3%	*n* = 3; 9%	0.618	NS
Moderate: >0.5 ≤ 1.0 AMD/PY	*n* = 2; 7%	*n* = 2; 6%	1.000	NS
Severe: >1.0 ≤ 2.0 AMD/PY	*n* = 6; 21%	*n* = 3; 9%	0.280	NS
Very severe: >2.0 AMD/PY	*n* = 11; 38%	*n* = 5; 15%	0.045	NS
Death: during or due to AMD	*n* = 3; 10%	*n* = 1; 3%	0.326	NS
NA	*n* = 3; 10%	*n* = 7; 21%	0.319	NS
Cognitive function				
Group 1: IQ >90 or regular education	*n* = 4; 14%	*n* = 15; 44%	0.013	NS
Group 2: IQ 60-90 or special education	*n* = 10; 34%	*n* = 8; 24%	0.407	NS
Group 3: IQ <60 or no education	*n* = 10; 34%	*n* = 5; 15%	0.081	NS
NA	*n* = 5; 17%	*n* = 6; 18%	1.000	NS
Mitochondrial complications				
None	*n* = 1; 3%	*n* = 5; 15%	0.205	NS
Mild	*n* = 6; 21%	*n* = 19; 56%	0.005	**0.034**
Moderate	*n* = 5; 17%	*n* = 5; 15%	1.000	NS
Severe	*n* = 9; 31%	*n* = 2; 6%	0.017	NS
Very severe	*n* = 8; 28%	*n* = 3; 9%	0.093	NS
Death	*n* = 0; 0%	*n* = 0; 0%	1.000	NS
NA	*n* = 0; 0%	*n* = 0; 0%	1.000	NS

Treatment-related complications	*Early onset (n = 29)*	*Late onset (n = 34)*	*P-value*	*Bonferroni correction*
None	*n* = 9; 31%	*n* = 17; 50%	0.199	NS
Mild	*n* = 10; 34%	*n* = 13; 38%	0.798	NS
Severe	*n* = 6; 21%	*n* = 3; 9%	0.280	NS
Very severe	*n* = 4; 14%	*n* = 1; 3%	0.171	NS
NA	*n* = 0; 0%	*n* = 0; 0%	1.000	NS

Adverse outcome of first sympt. phase: Adverse outcome of the first symptomatic phase. NA: not assessed; NS: not significant. Early onset: presentation ≤28 days of life; Late onset: presentation >28 days of life. Statistical significance was determined by fisher's exact tests. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold.

Table 3. Expected effect of NBS implementation on occurrence of adverse outcomes due to the first symptomatic phase.

Patients	Group	ID	Formula	Day 0 (% (n))	Day 3 (% (n))	Day 7 (% (n))	Day 11 (% (n))	Day 15 (% (n))	Day 21 (% (n))
Potentially diagnosed through NBS (after NBS implementation)	Total	A,a		100% (61)	84% (51)	61% (37)	56% (34)	54% (33)	54% (33)
	Without AO	B,b	A-C, a-c	54% (33)	59% (30)	65% (24)	68% (23)	70% (23)	70% (23)
	With AO	C,c	A-B, a-b	46% (28)	41% (21)	35% (13)	32% (11)	30% (10)	30% (10)
Potentially diagnosed after first symptomatic phase (after NBS implementation)	Total	D,d		0% (0)	16% (10)	39% (24)	44% (27)	46% (28)	46% (28)
	Without AO	E,e	D-F, d-f	0% (0)	30% (3)	38% (9)	37% (10)	36% (10)	36% (10)
	With AO	F,f	D-E, d-e	0% (0)	70% (7)	62% (15)	63% (17)	64% (18)	64% (18)
Index siblings of patients identified through family testing	Total	G,g		100% (9)	100% (9)	100% (9)	100% (9)	100% (9)	100% (9)
	Without AO	H.h		11% (1)	11% (1)	11% (1)	11% (1)	11% (1)	11% (1)
	With AO	I,i		89% (8)	89% (8)	89% (8)	89% (8)	89% (8)	89% (8)
Siblings identified through family testing	Without AO	J,j		56% (5)	56% (5)	56% (5)	56% (5)	56% (5)	56% (5)
	With AO	K,k		44% (4)	44% (4)	44% (4)	44% (4)	44% (4)	44% (4)
Estimated effectivity Protected from AO (after NBS implementation)		L m	k / i L * c	50% *n* = 14	50% *n* = 11	50% *n* = 7	50% *n* = 6	50% *n* = 5	50% *n* = 5
Percentage AO first symptomatic phase (after NBS implementation)		N,n	n / (a+d) c + f - m	**23%** ***n* = 14**	**28%** ***n* = 17**	**34%** ***n* = 21**	**36%** ***n* = 22**	**38%** ***n* = 23**	**38%** ***n* = 23**

AO: adverse outcome; ID: identifier; NBS: newborn screening. Total number of patients included is 61; excluding patients identified through family testing *n* = 12, excluding patients for whom the exact day of diagnosis is unknown, *n* = 3 (Figure 1). The column listing the identifiers indicates the identifier of the percentage in capitals, or the number of patients in lowercases, for the interpretation of the formula. The formula indicates how the percentages and numbers of patients were calculated. The five time points indicate the days that NBS results could become available when NBS would be implemented. All percentages and patient numbers were rounded.

3.3 | AMD frequency

In total, 962 AMD requiring hospital admission were reported. Hospitalization duration was significantly shorter in MMA than in PA patients. AMD triggers were similar, with the exception of significantly more frequent feeding problems in MMA patients and constipation in PA patients (Table S6).

Among the sibling pairs, AMD frequency was similar in 4/9 pairs. The index patients of 4/9 sibling pairs died following an AMD, whereas all but one of the siblings identified through family testing are still alive, although currently only P6.3 survived his sibling in age (Table S1). AMD frequency tended to be shifted towards a more severe frequency in EO patients compared to LO patients (Table 2, Figure S1). Between PA and MMA patients no difference in AMD frequency was recorded. (Table S6). Among PA patients, EO patients experienced significantly more AMD/PY ($p = 0.002$) and among MMA patients, vitamin B12 unresponsive patients experienced significantly more AMD/PY (Table S6).

3.4 | Cognitive function

A total of 139 neuropsychological test results were recorded in 46/76 patients, with a median of 3 tests per patient (range: 1 – 10). In 17 patients, cognitive function was based on the patients' educational level and in 13 patients, cognitive function was not assessed (yet). An IQ >90 was noted in 24/63 patients, an IQ between 60 and 90 in 20/63 patients and an IQ <60 in 19/63 patients (Table S7).

Cognitive function was comparable among the sibling pairs (similar in 3, better in 2, worse in 1, not assessed in 3) (Table S1), as well as between EO and LO patients (Table 2, Figure S1). An IQ <60 was significantly more prevalent in PA patients (Table S7). Among PA patients, EO was significantly associated with cognitive function (IQ >90: EO 0%, LO 63%, $p = 0.002$). Among MMA patients, presentation type nor vitamin B12 responsiveness were associated with cognitive function.

3.5 | Complications with potential mitochondrial etiology

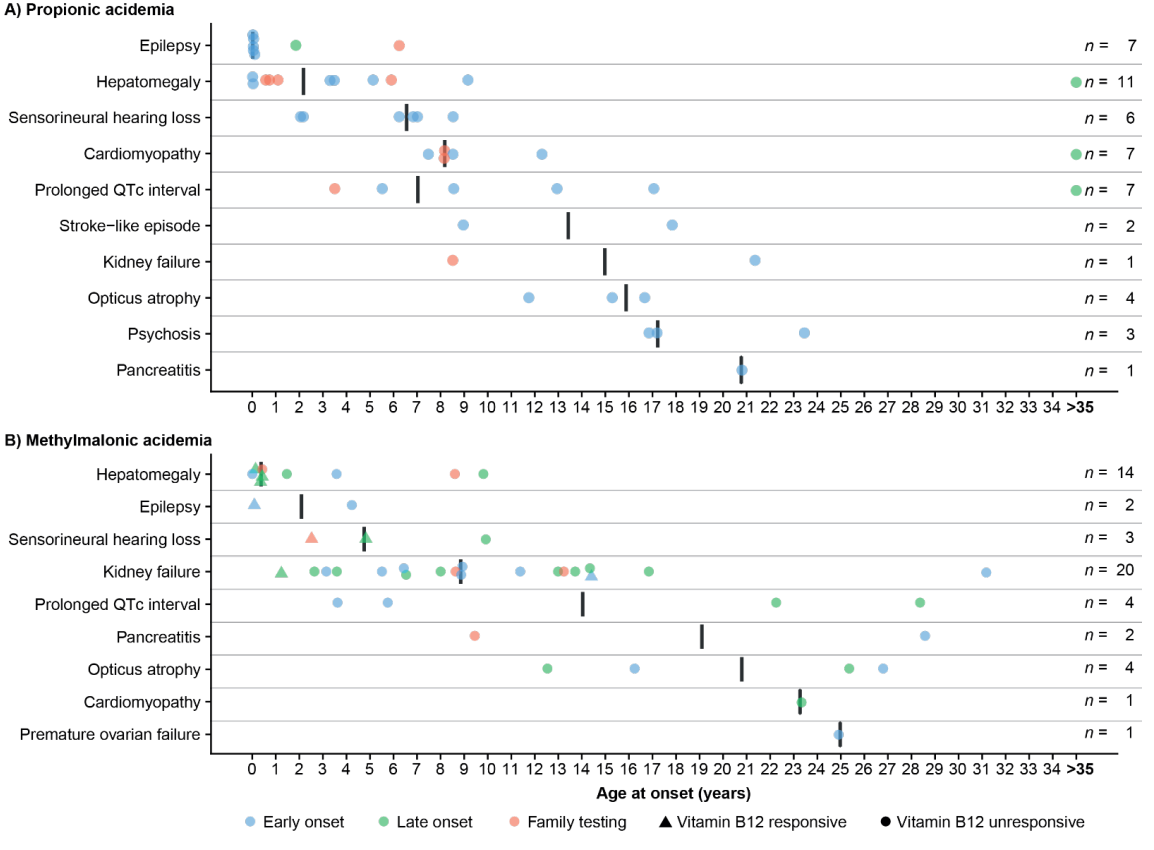
Mitochondrial complications were present in 70/76 patients, and were mild in 30, moderate in 13, severe in 12 and very severe in 14 patients. One PA patient with very severe mitochondrial complications died due to cardiomyopathy. Prevalence of mitochondrial

complications was compared with the literature and was fairly comparable for most complications [2] (Table S7). For quite some complications, especially for MMA, prevalence was here assessed for the first time in a cohort study (Table S7). Renal failure was significantly more prevalent among MMA patients than among PA patients (Table S7).

For mitochondrial complications with acute onset, age at onset was assessed (Table S7, Figure 2). The earliest manifestation of renal failure was already reported at the age of 1.2 years in an *MMAB* patient and in 5/20 patients age of onset was < 6 years (2 *MUT,* 2 *MMAB*, one unclassified). The first manifestation of prolonged QTc interval was shortly after birth in PA, and at 3.6 years in MMA. Cardiomyopathy in PA was noted at 7.5 years, optic atrophy at 11.8 years in PA and at 12.6 years in MMA (Table S7). Age at onset of hepatomegaly, epilepsy and sensorineural hearing loss ranged from birth to ten years, while most other complications typically arose from late infancy to adolescence (Table S7, Figure 2). Mitochondrial complications were comparable among sibling pairs (similar in 1, better in 4, worse in 4) (Table S1). LO patients were significantly more frequently recorded to have only mild mitochondrial complications than EO patients (Table 2, Figure S1). PA patients had more very severe mitochondrial complications than MMA patients (Table S7), and all of these PA patients were EO patients (EO 53%, LO 0%, p = 0.019). Among MMA patients, mitochondrial complications were comparable between EO and LO patients but the prevalence of moderate to very severe mitochondrial complications was significantly higher in vitamin B12 unresponsive patients (unresponsive 63%, responsive 24%, p = 0.016).

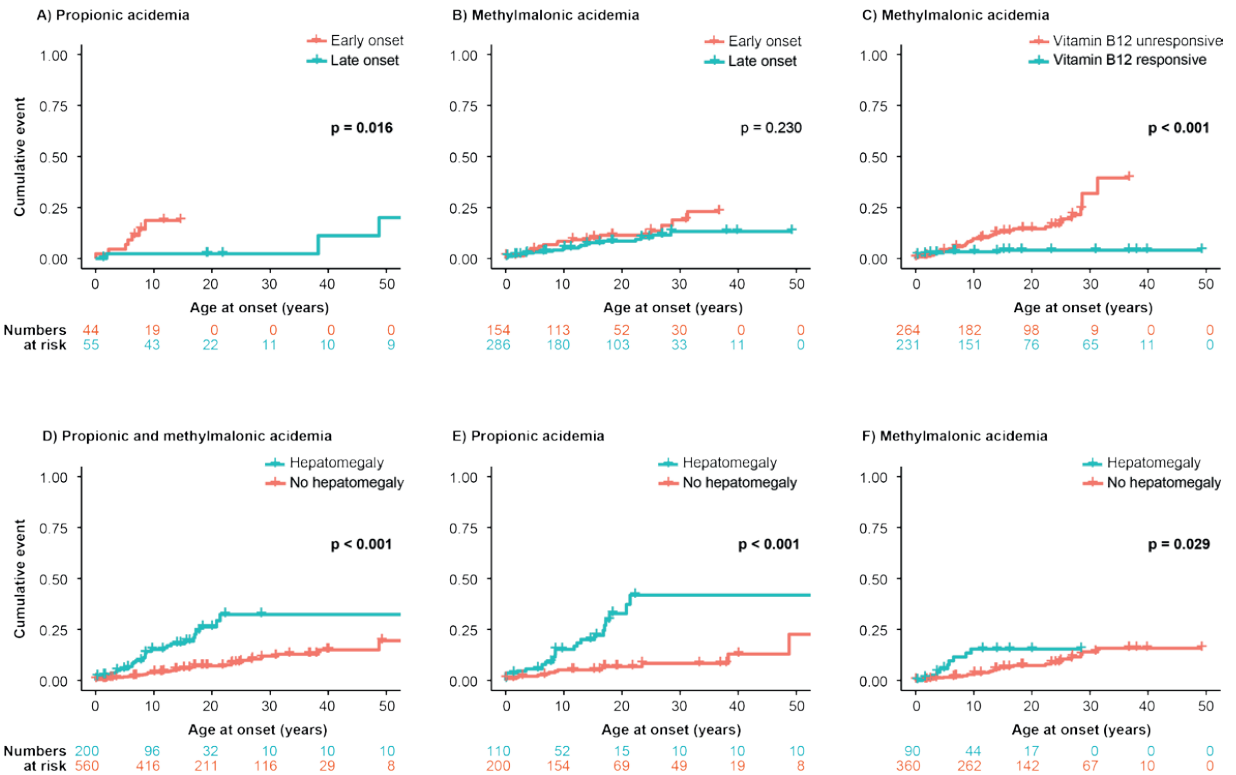
EO for PA and vitamin B12 unresponsiveness for MMA were also found to be significantly associated with a higher risk for mitochondrial complications with acute onset (Figure 3). In addition, EO in PA tended to be associated with the occurrence of hepatomegaly, prolonged QTc interval, psychoses and sensorineural hearing loss (Figure S2). Vitamin B12 unresponsiveness tended to be associated with the occurrence of optic atrophy, pancreatitis and prolonged QTc interval in MMA, and was significantly associated with the occurrence of renal failure (Figure S3). One of the first occurring mitochondrial complications with acute onset was hepatomegaly (Figure 2). Intriguingly, hepatomegaly was significantly associated with the onset of other mitochondrial complications with acute onset later in life, for PA and MMA as a group and for both PA and MMA separately (Figure 3).

FIGURE 2. Age at onset of mitochondrial complications with acute onset.



Panel A depicts propionic acidemia, panel B depicts methylmalonic acidemia. The y-axis depicts the mitochondrial complications with acute onset, ordered on median age at onset. The x-axis depicts patient age in years, with a maximum of 35 years of age. For all ages > 35 years, the circles have been placed at 35 years. The black vertical lines depict the median age at onset. The filled circles and triangles depict the individual patient age at onset of a complication. Circles depict no vitamin B12 responsiveness, triangles depict vitamin B12 responsiveness of methylmalonic acidemia patients. Blue circles and triangles depict early onset, green circles and triangles depict late onset, orange circles and triangles depict diagnosis through family testing. Early onset: presentation ≤28 days of life; late onset: presentation >28 days of life.

FIGURE 3. Factors associated with increased risks for mitochondrial complications with acute onset.



Kaplan-Meier plots wherein the y-axis depicts the cumulative percentage and the x-axis depicts patient age in years. Numbers at risk for the subgroups, indicating for how many complications the patients are at risk, are depicted below the panels, in corresponding colors. Panel A-B demonstrate late onset in blue versus early onset in orange for propionic acidemia (PA) in panel A and for methylmalonic acidemia (MMA) in panel B. Early onset: presentation ≤28 days of life; late onset: presentation >28 days of life. Panel C demonstrates vitamin B12 responsiveness in blue versus vitamin B12 unresponsiveness in orange for MMA. Panel D-F depicts the association of the presence of hepatomegaly to other mitochondrial complications with acute onset in blue, versus no hepatomegaly in orange, in panel D for both PA and MMA, in panel E for PA and in panel F for MMA.

3.6 | Complications with potential treatment-related etiology

Reduced BMD was noted in 27, growth retardation in 21 and obesity in 16 patients. Treatment-related complications were absent in 33, mild in 28, severe in 9 and very severe in 6 patients (Table S8).

Treatment-related complications were comparable among sibling pairs (similar in 2, better in 5, worse in 2) (Table S1), as well as among EO and LO patients (Table 2, Figure S1), nor were there any differences between PA and MMA, or among PA patients between EO and LO patients (Table S8). Among MMA patients, the rate of mild, severe and very severe treatment-related complications was significantly higher in vitamin B12 unresponsive patients (unresponsive 71%, unresponsive 33%, $p = 0.017$).

3.7 | Complications with miscellaneous etiology

A prevalence of 29% pes planovalgus, a feature so far only described in MMA[18], was noted in PA. In one MMA and one PA patient urolithiasis was recorded. In both patients, calculi developed due to hypercalciuria, although dehydration, chronic acidotic state and a low-protein diet might have contributed. Two MMA patients (*MUT* and *MMAA*) were found to have juvenile gout (Table S8). The *MMAA* patient had chronic renal failure when exhibiting gout, whereas the *MUT* patient did not.

4 | Discussion

4.1 | Expected health gain of NBS for PA and MMA in the Netherlands (WHO#4)

Here we present an extensive, nationwide cohort study, describing the clinical course of 76/83 Dutch PA and MMA patients. We demonstrated that the expected health gain of introducing NBS for PA and MMA in the Netherlands in overall outcome may be limited. Patients can already present shortly after birth and even if patients are diagnosed pre-symptomatically, they can still develop an adverse outcome. We calculated an expected reduction from 46% to 36-38% AO due to the first symptomatic phase and we consider it unlikely that NBS could result in reduced AMD frequency, improved cognitive function or a reduced frequency of mitochondrial and therapy-related complications.

These findings are in line with previous reports. Although time to diagnosis is reduced in patients identified by NBS [9], NBS is unlikely to identify more than 59-72% of PA and MMA patients before the first symptomatic phase [8, 9]. One study described no decreased AMD frequency, no better neurological outcome and no reduced frequency of long-term complications in PA patients [8], although another study found that PA patients identified by NBS had lower cardiac manifestations [9], but follow-up time in these patients was limited. In MMA patients, besides improved attainment of motor milestones and reduced manifestations of movement disorders in vitamin B12 unresponsive patients, NBS implementation did not result in other improved outcome parameters [9].

Thus, as evidence supporting improved outcomes if treatment can be initiated in the asymptomatic phase is limited, despite reasonable consensus on therapeutic strategies for PA and MMA [18, 19], it is questionable whether the criterion regarding an accepted (effective) treatment for patients with recognized disease is met (W&J#2). As a consequence, it remains debatable whether the criteria requesting effectiveness (WHO#4) and benefits outweigh harm (WHO#10), are met (Supplementary Notes 1). We advocate that the (now limited) health gain of NBS can only be improved if treatment strategies are identified that can effectively prevent AMD, cognitive impairment and mitochondrial complications, such as innovative therapies aiming to increase enzyme activity [19].

4.2 | Evaluation of the implementation of NBS for PA and MMA (WHO #9)

Insight in NBS efficacy is still limited, due to factors complicating proper evaluation, such as timing of evaluation. Follow-up time of the four studies describing the potential additional value of NBS for PA and MMA was relatively short, resulting in uncertainty when drawing conclusions [6-9]. We propose that these countries reassess their NBS cohorts when a longer follow-up time is available. For the Netherlands, assuming a similar incidence in the NBS cohort as in the here presented cohort, but taking into account the slightly decreasing birth rate, it will take at least till 2050 before an NBS cohort similar in size and follow-up time as the pre-NBS cohort is available for evaluation. More timely evaluation can only be achieved by composing larger NBS cohorts through data sharing initiatives, within Europe or worldwide.

Even if timing of evaluation is adequate, proper evaluation of the presumed effect of NBS remains challenging given the many factors that can influence the clinical course of PA and MMA. This includes genotypic factors as mutation type, residual enzyme activity and vitamin B12 responsiveness (MMA). Also, a high variability in clinical management existed between the different metabolic centers and metabolic physicians (data not shown). Next, compliance of parents and patients to clinical management, and disease insight could influence clinical outcomes. Moreover, pre-NBS cohorts are diagnosed and treated in the era before NBS cohorts, with NBS cohorts taking advantage of general advances in clinical care, and possibly increased insights and improved therapies for PA and MMA patients.

To aid evaluation of NBS efficacy, we suggest the five clinical outcome parameters here presented and we advocate the introduction of continuation and stop criteria. We propose that NBS for PA and MMA should be continued when it results in a statistically significant and clinically relevant reduction of AO due to the first symptomatic phase, and that it should be stopped otherwise. While based on our results, NBS may be unlikely to result in reduced AMD frequency, improved cognitive function and reduced mitochondrial complications, these outcome parameters should be assessed to gain insight in the effects of improved treatment strategies. If NBS would unexpectedly result in improvement of these parameters, this would be a supportive argument to continue NBS. NBS evaluation should also include assessment of treatment-related complications, to gain knowledge regarding risks and harm of screening.

In NBS cohorts, patients in whom the disease is mild and who would not become symptomatic during infancy, could be detected as has been reported in Japan, where a ten times higher incidence of PA was found during preparatory studies for NBS implementation [20]. These mildly affected patients will probably be treated according to the current treatment guidelines [18], inducing a risk for developing treatment-related complications. One could advocate that these patients should not be treated in order to prevent treatment-related complications. However, in our cohort, two PA patients presented at 48 and 56 years of age with cardiomyopathy as first presenting symptom [21], indicating that even in very mild, untreated patients, mitochondrial complications can still occur. It is presently unknown whether earlier initiation of adequate treatment could have prevented this complication. Even if this would alter the clinical course, it is questionable whether these patients should be exposed to life-long protein restriction, to prevent complications occurring well into adulthood. We consider overtreatment of mildly affected patients, that might or might not develop late complications [20, 21], a serious risk of NBS.

4.3 | Lessons learned from the pre-NBS cohort, to guide follow-up of post-NBS cohorts

We demonstrated that mitochondrial complications with acute onset can already develop before monitoring should be initiated according to recent guidelines [18] and we thus propose to adapt these guidelines. Specifically, we suggest to assess renal function in *MUT* and *MMAB* patients from one year of age, to regularly check QTc intervals in PA and MMA patients starting at birth, and to maintain yearly cardiac ultrasounds and ophthalmologic assessments from six years onwards. Importantly, as we demonstrate that hepatomegaly is associated with the occurrence of other acute onset mitochondrial complications later in life, we advise to perform physical examination to check for hepatomegaly during every outpatient visit starting at birth, to be able to study the relevance of this finding. Lastly, we advocate awareness of all complications assessed in this cohort, including rarely described complications as exercise intolerance, acute psychosis, premature ovarian failure, pes planovalgus, gout and urolithiasis.

For surveillance and counseling of patients in NBS cohorts, we stress the importance to take into account disease severity. For MMA patients we demonstrated that vitamin B12

unresponsiveness increases the risk of AMD frequency, mitochondrial complications and treatment-related complications. This is in line with previous reports, that demonstrate that vitamin B12 unresponsiveness is associated with a decreased survival rate in EO patients, a higher frequency of AMD, an increased rate of developmental delay in EO patients and an increased rate of disability [22]. In addition, a tendency towards an increased rate of chronic renal failure has been reported [23]. For PA patients, EO is associated with a higher risk for an AO of the first symptomatic phase, AMD frequency, cognitive impairment and mitochondrial complications. While this still holds true for patients that are symptomatic before NBS results are available, presentation type will be unknown for PA patients diagnosed via NBS, complicating surveillance and counseling of these patients. This augments the need for systematic determination of PCC activity in PA patients and illustrates the imminent need for a metabolic marker that can predict the risks for AMD frequency, cognitive function and mitochondrial complications [24, 25].

We could not identify specific genotype-phenotype correlations due to the variation of the identified mutations. However, we did note that whereas patients with *MMAB* type MMA are generally reported to be vitamin B12 unresponsive [18], 6/7 of our *MMAB* patients were responsive. In 5 of these, we identified the previously unreported *MMAB* c.556C>T missense mutation in either homozygous or heterozygous fashion (Table S2). We speculate that this mutation might be related to the observed vitamin B12 responsiveness of *MMAB* patients, but we cannot prove this hypothesis.

4.4 | Limitations and strengths

We note a few limitations. First, due to the retrospective nature of this study and variable follow-up schedules, missing data could have affected the reliability of the analyses. We expect missing data to be random and consider it unlikely to have resulted in confounding. Nevertheless, to facilitate reliable analyses and conclusions on NBS effectivity, we advocate precise and structured prospective follow-up of NBS cohorts. Minimum requirements for evaluation are listed in Table S9. Centers participating in data-sharing initiatives should agree on the items to be recorded and on the timing of diagnostics studies (Table S9). Second, sample size of the sibling comparison is limited, hampering solid conclusions on the expected health gain of NBS for PA and MMA, but consolidating the urge for thorough

assessment of NBS cohorts. The potential health gain might have been overestimated 1) due to the unknown number of EO patients that died undiagnosed, 2) since families with an index child are prepared for the diagnosis, and can minimize the risk for AMD more efficiently than families from individuals identified by NBS, 3) since calculations were performed with a potential overestimation of AO due to the first symptomatic phase, as we assumed that all observed brain imaging abnormalities were caused by the first symptomatic phase instead of being caused by AMD occurring later in life (and thus under treatment). Conversely, the potential health gain of NBS might have been underestimated as the clinical course between siblings may vary and it is not known whether the sibling diagnosed through family testing would have stayed in the same condition if diagnosed later in life.

Important strengths of our study are that we provide a comprehensive overview of a nationwide cohort spanning over four decades, by systematically assessing nearly all PA and MMA patients in the Netherlands in great detail. We designed five clinical outcome parameters to guide evaluation of implementation NBS and we provide an extensive description of a pre-NBS cohort to compare NBS cohorts with. Moreover, due to the systematic assessment of AMD, complications and risk factor analysis, we were able to establish recommendations regarding surveillance and counseling of NBS cohorts. Lastly, continuation and stopping criteria for NBS were provided, as well as minimum requirements for follow-up of NBS cohorts to facilitate data sharing initiatives.

5 | Conclusion

The objective of NBS for PA and MMA in the Netherlands is to protect LO patients from irreversible neurological damage due to the first symptomatic phase. Implementation in the Netherlands may result in a reduction of AO due to the first symptomatic phase from 46% to 36-38%, if NBS results become available between day 11 and 21. We consider reduced AMD frequency, improved cognitive function and reduced mitochondrial and treatment-related complications unlikely given the currently available therapies.

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Supplemental tables and figures

Supplementary Notes 1 – Screening criteria and recommendations

1968, Wilson and Jungner, ten criteria to guide selection of conditions suitable for screening

(1) The condition sought should be an important health problem.
(2) There should be an accepted treatment for patients with recognized disease.
(3) Facilities for diagnosis and treatment should be available.
(4) There should be a recognizable latent or early symptomatic stage.
(5) There should be a suitable test or examination.
(6) The test should be acceptable to the population.
(7) The natural history of the condition, including development from latent to declared disease, should be adequately understood.
(8) There should be an agreed policy on whom to treat as patients.
(9) The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.
(10) Case-finding should be a continuing process and not a "once and for all" project.

2008, World Health Organization, ten criteria additional to the Wilson and Jungner criteria

(1) The screening programme should respond to a recognized need.
(2) The objectives of screening should be defined at the outset.
(3) There should be a defined target population.
(4) There should be scientific evidence of screening programme effectiveness.
(5) The programme should integrate education, testing, clinical services and programme management.
(6) There should be quality assurance, with mechanisms to minimize potential risks of screening.
(7) The programme should ensure informed choice, confidentiality and respect for autonomy.
(8) The programme should promote equity and access to screening for the entire target population.
(9) Programme evaluation should be planned from the outset.
(10) The overall benefits of screening should outweigh the harm.

Supplementary Notes 2 – Definition of grouped outcome parameters

Definition of "Adverse outcome (AO) of the first symptomatic phase" as clinical outcome parameter

No AO: No basal ganglia hyperintensities, or white matter abnormalities or movement disorder

Mild AO: Basal ganglia hyperintensities or white matter abnormalities or movement disorder that can be most likely attributed to the first symptomatic phase, but not wheelchair-bound

Severe AO: Basal ganglia hyperintensities or white matter abnormalities or movement disorder, wheelchair-bound due to movement disorders that most likely arose due to the first symptomatic phase

Death: Death during or due to the first symptomatic phase

Definition of "AMD frequency" as clinical outcome parameter

None: No episode of AMD during first four years of life

Mild: >0.0 ≤ 0.5 AMD/PY during first four years of life

Moderate: >0.5 ≤ 1.0 AMD/PY during first four years of life

Severe: >1.0 ≤ 2.0 AMD/PY during first four years of life

Very severe: >2.0 AMD/PY during first four years of life

Death: Death during or due to AMD, at any moment in life

Not assessed (NA): *if death during or due to first presentation*

Definition of "Cognitive function" as clinical outcome parameter

Group 1: IQ >90 or regular education

Group 2: IQ 60-90 or special education

Group 3: IQ <60 or no education

Not assessed (NA): *Last moment of follow-up <4 years of age*

Definition of "Mitochondrial complications" as clinical outcome parameter

Twenty-one complications considered to be potentially caused by mitochondrial failure based on an extensive literature review[2] were included: hepatomegaly, epilepsy, cardiomyopathy, optic atrophy, pancreatitis, renal failure, sensorineural hearing loss, acute psychosis, stroke-like episodes, prolonged QTc interval, premature ovarian insufficiency, exercise intolerance, autism, feeding disorders, muscular hypotonia, constipation, attention deficit hyperactive disorder, anemia, leukopenia, thrombocytopenia and pancytopenia.

Severity is based on the number of complications recorded at the last moment of follow-up, as follows:

	0-12 year	13-18 year	19-24 year	>25 years
None:	0	0	0	0
Mild:	1-2	1-4	1-6	1-8
Moderate:	3-4	5-6	7-8	9-10
Severe:	5-6	7-8	9-10	11-12
Very severe:	≥7	≥9	≥11	≥13

Death: Death due to mitochondrial complication

Not assessed (NA): *If death during or due to first presentation*

Definition of "Treatment-related complications" as clinical outcome parameter

None: No decreased BMD, no growth retardation, no obesity

Mild:	1 of the following: decreased BMD, growth retardation, obesity
Severe:	2 of the following: decreased BMD, growth retardation, obesity
Very severe:	Decreased BMD and growth retardation and obesity

Not assessed (NA): *If death during or due to first presentation*

Supplementary table 1 – Comparison of baseline characteristics and outcome parameters of patients identified through family screening and their index siblings

	P1.1	P1.2	P2.1	P2.2	P3.1	P3.2	P4.1	P4.2	P5.1	P5.2	P6.1	P6.2	P6.3
Gene	*PCCA*	*PCCA*	*PCCB*	*PCCB*	*PCCB*	*PCCB*	PA, uncl	PA, uncl	PA, uncl	PA, uncl	PA, uncl	PA, uncl	PA, uncl
Sex	M	F	M	M	M	M	F	M	M	F	F	M	M
Age at diagnosis	Day 15	Day 0	0.7 y	Day 2	Day 3	Day 0	Day 18	Day 1	5.0 y	2.0 y	Day 8	Day 4	Day 1
Age at death (years)		8.6	19.1		7.0		12.1				2.9	1.4	
Age last follow-up (years)	18.4	8.6	19.1	19.0	7.0	5.6	12.1	9.7	37.0	33.4	2.9	1.4	11.4
Presentation type	EO	Family	LO	Family	EO	Family	EO	Family	LO	Family	EO	Family	Family
Adverse outc. first sympt. phase	Mild	No	Severe	No	Mild	No	Mild	Mild	No	No	Mild	Mild	Mild
AMD frequency	Severe	Severe	Death	Stable	Death	V. severe	Death	Severe	Stable	Stable	Death	Death	V. severe
Cognitive function	Group 3	Group 3	Group 3	Group 1	NA	Group 3	Group 2	Group 3	Group 1	Group 1	NA	NA	Group 3
Mitochondrial complications	V. severe	Death	Moderate	Mild	V. severe	Severe	Severe	V. severe	None	Mild	V. severe	V. severe	V. severe
Treatment-related complications	V. severe	Mild	Mild	Mild	None	Mild	V. severe	None	None	None	None	None	Mild

	P7.1	P7.2	P8.1	P8.2	P9.1	P9.2
Gene	*MUT*	*MUT*	*MUT*	*MUT*	*MMAB*	*MMAB*
Sex	M	M	M	F	F	M
Age at diagnosis	Day 3	Day 0	0.2 y	Day 0	3.9 y	1.6 y
Age at death						
Age last follow-up	13.9	1.7	18.5	13.7	18.3	16.1
Presentation type	EO	Family	LO	Family	LO	Family
Vitamin B12 responsiveness	No	No	No	No	Yes	Yes
Adverse outc. first sympt. phase	Mild	Mild	Mild	Mild	Severe	No
AMD frequency	Severe	NA	Severe	Severe	Stable	Stable
Cognitive function	Group 3	NA	Group 3	Group 2	Group 1	Group 1
Mitochondrial complications	Severe	Moderate	Severe	Moderate	None	Mild
Treatment-related complications	V. severe	Mild	V. severe	Mild	Mild	None

Adverse outc. first sympt. phase: Adverse outcome of the first symptomatic phase. AMD: acute metabolic decompensation; M: male; F: female; NA: Not assessed; EO: early onset; LO: late onset; Family: family testing; V. severe: very severe. Early onset: presentation $\leq$28 days of life; Late onset: presentation >28 days of life. Cognitive function group 1: IQ >90 or regular education, group 2: IQ 60-90 or special education, group 3: IQ <60 or no education.

Supplementary table 2 – All mutations detected in PA and MMA related genes in the Dutch patient cohort

Gene	Mutation (c.)	Mutation (p.)	Mutation type	Reported	Alleles	Origin	Remarks
PCCA	c.625G>C	p.Ala209Pro	Missense/Nonsense	No	1	Dutch	
PCCA	c.923dup	p.Leu308Phefs*35	Duplication	No	1	Dutch	
PCCA	c.1409T>G	p.Leu470Arg	Missense/Nonsense	No	6	Moroccan	2 siblings HO, not reported consanguine; 1 individual HO, consanguine, not knowingly related to the 2 siblings
PCCA	c.2077A>C	p.Met693Leu	Missense/Nonsense	No	1	Dutch	
PCCA	Exon 2 deletion		Gross deletion	No	1	Dutch	
PCCA	Exon 5 and 6 deletion		Gross deletion	No	1	Dutch	
PCCA	Exon 10 deletion		Gross deletion	No	1	Dutch	
PCCA	c.1900-1G>A		Splicing	Yang et al. 2004	2	Afghan	HO, consanguine
PCCA	c.2127delT	p.Val710Cysfs*43	Small deletion	Campeau et al. 1999	1	Dutch	
PCCB	c.644G>C	p.Gly222Arg	Missense/Nonsense	No	2	Turkish	HO, consanguine
PCCB	c.671C>T	p.Ala224Val	Missense/Nonsense	No	1	Dutch	
PCCB	c.703A>C	p.Thr235Pro	Missense/Nonsense	No	4	Moroccan	2 siblings, HO, not reported consanguine
PCCB	c.883_885del	p.Phe295del	Small deletion	No	1	Dutch	
PCCB	c.1127G>A	p.Arg376His	Missense/Nonsense	No	4	Turkish	2 siblings, HO, consanguine
PCCB	c.337C>T	p.Arg113*	Missense/Nonsense	Brosch et al. 2008	2	Turkish	HO, consanguine
MUT	c.623_624del		Small deletion	No	1	Dutch	
MUT	c.730A>C	p.Gln213His	Missense/Nonsense	No	2	Dutch	HO, consanguinity unknown
MUT	c.1022dupA	p.Asn341fs*	Duplication	No	1	Surinamese	
MUT	c.1280G>T	p.Gly427Val	Missense/Nonsense	No	2	Egyptian	HO, consanguine
MUT	c.1311_1312insA	p.Val438Serfs*3	Small insertion	No	4	Turkish/Dutch	1 individual HO, consanguine; 1 individual HO, consanguinity unknown, not knowingly related
MUT	c.1690G>T	p.Glu564*	Missense/Nonsense	No	2	Turkish	HO, consanguine
MUT	c.1962_1963delTC	p.Arg655*	Missense/Nonsense	No	4	Turkish	2 siblings, HO, consanguine
MUT	c.2078delG	p.Gly693Aspfs*12	Small deletion	No	2	Moroccan	HO, consanguine
MUT	c.322C>T	p.Arg108Cys	Missense/Nonsense	Worgan et al. 2006	4	Dutch	HE, 2 siblings, 2 individuals, not knowingly related
MUT	c.454C>T	p.Arg152*	Missense/Nonsense	Martínez et al. 2005	3	Turkish/Dutch	1 individual HO, consanguine; 1 individual HE, not knowingly related
MUT	c.654A>C	p.Gln218His	Missense/Nonsense	Fuchshuber et al.	6	Dutch	2 individuals HO, 2 individuals HE, not reported

				2000			consanguine, not knowingly related
MUT	c.655A>T	p.Asn219Tyr	Missense/Nonsense	Acquaviva et al. 2005	7	Turkish/Dutch	2 individuals HO (1 consanguine); 3 individuals (2 siblings) HE, not knowingly related
MUT	c.1106G>A	p.Arg369His	Missense/Nonsense	Janata et al. 1997	3	Turkish/Dutch	1 individual HO, consanguine; 1 individual HE, not knowingly related
MUT	c.1160C>T	p.Thr387Ile	Missense/Nonsense	Dündar et al. 2012	2	Syrian	HO, consanguine
MUT	c.1531C>T	p.Arg511*	Missense/Nonsense	Acquaviva et al. 2005	1	Dutch	
MUT	c.1677-1G>C		Splicing	Acquaviva et al. 2005	1	Dutch	
MUT	c.2150G>T	p.Gly717Val	Missense/Nonsense	Crane et al. 1992	1	Surinamese	
MMAA	c.202C>T	p.Gln68*	Missense/Nonsense	No	1	Dutch	
MMAA	c.455del	p.Pro152Leufs*9	Small deletion	No	6	Turkish	2 siblings, 1 cousin, HO, consanguine
MMAA	c.433C>T	p.Arg145*	Missense/Nonsense	Yang et al. 2004	5	Dutch	2 individuals HO (1 consanguine); 1 HE, not knowingly related
MMAA	c.733+1G>A		Splicing	Lerner-Ellis et al. 2004	4	Dutch	1 individual HO (not reported consanguine); 2 individuals HE, not knowingly related
MMAB	c.556C>T	p.Arg186Trp	Missense/Nonsense	No	5	Dutch	1 individual HO (not reported consanguine); 3 individuals HE (2 siblings), patients not knowingly related (except the siblings)
MMAB	c.565_577del	p.Cys189Argfs*	Small deletion	No	1	Hindi	
MMAB	c.655T>C	p.Tyr219His	Missense/Nonsense	No	1	Hindi	
MMAB	c.569G>A	p.Arg190His	Missense/Nonsense	Lerner-Ellis et al. 2006	1	Dutch	
MMAB	c.197-1G>A		Splicing	Lerner-Ellis et al. 2006	2	Dutch	HE, 2 siblings
MMAB	c.700C>T	p.Gln234*	Missense/Nonsense	Lerner-Ellis et al. 2006	2	Dutch	HE, 2 individuals, not knowingly related

Mutation (c.) depicts the genetic change in the DNA coding sequence, mutation (p.) depicts the resulting change in the protein coding sequence. HO: homozygous, HE: heterozygous. In one patient with a mutation in *PCCA,* no other mutation at the other allele was identified. References:

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Supplementary table 3. Pregnancy, delivery and birth weight parameters.

	Propionic acidemia (*n* = 31)	Methylmalonic acidemia (*n* = 45)	P-value	Bonferroni correction
Maternal health problems during pregnancy	*n* = 2; 6%	*n* = 3; 7%	1.000	NS
Delivery via caesarean section	*n* = 4; 13%	*n* = 2; 4%	0.218	NS
Gemelli	*n* = 1; 3%	*n* = 1; 2%	1.000	NS
Gestational age				
Prematurity (<36 weeks)	*n* = 0; 0%	*n* = 5; 11%	0.075	NS
Serotinity (>42 weeks)	*n* = 0; 0%	*n* = 5; 11%	0.075	NS
Abnormal APGAR scores	*n* = 2; 6%	*n* = 4; 9%	1.000	NS
Birth weight (median ± SD; (n))	0.38 ± 1.61 SDS (23)	-1.48 ± 1.19 SDS (32)	<0.001	**<0.001**
Birth weight < -2 SDS	*n* = 2; 9% (23)	*n* = 10; 31% (32)	0.055	NS

SDS: standard deviation score; NS: not significant. Student's t-tests were performed for quantitative data and Fisher's exact tests were performed for qualitative data. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold.

Supplementary table 4. Symptoms and signs, laboratory tests and interventions during the first symptomatic phase.

		Early onset (*n* = 29)	Late onset (*n* = 34)	P-value	Bonferroni correction
Symptoms and signs					
Lethargy		*n* = 21; 72%	*n* = 19; 56%	0.199	NS
Anorexia		*n* = 20; 69%	*n* = 18; 53%	0.211	NS
Vomiting		*n* = 6; 21%	*n* = 26; 76%	<0.001	**<0.001**
Hypotonia		*n* = 14; 48%	*n* = 12; 35%	0.318	NS
Dehydration		*n* = 11; 38%	*n* = 8; 24%	0.275	NS
Kussmaul breathing		*n* = 9; 31%	*n* = 10; 29%	1.000	NS
Tachypnea		*n* = 9; 31%	*n* = 2; 6%	0.017	NS
Weight loss		*n* = 5; 17%	*n* = 5; 15%	1.000	NS
Hypothermia		*n* = 7; 24%	*n* = 1; 3%	0.019	NS
Coma		*n* = 1; 3%	*n* = 5; 15%	0.205	NS
Failure to thrive		*n* = 0; 0%	*n* = 6; 18%	0.027	NS
Global developmental delay		*n* = 0; 0%	*n* = 3; 9%	0.243	NS
Laboratory tests		*Median ± SD [range] (N)*	*Median ± SD [range] (N)*		
Glucose	mmol/L	5.5 ± 6.2 [1.6 – 25.4] (22)	7.2 ± 7.6 [1.2 – 34.0] (23)	0.465	NS
pH		7.33 ± 0.14 [6.89 – 7.43] (26)	7.28 ± 0.17 [6.81 – 7.42] (27)	0.179	NS
pCO_2	mmHg	28.1 ± 14.0 [12.0 – 62.3] (23)	18.7 ± 12.5 [10.0 – 60.0] (22)	0.023	NS
Bicarbonate	mmol/L	16.1 ± 6.3 [3.2 – 24.9] (25)	7.5 ± 6.7 [2.8 – 23.0] (22)	0.006	NS
Base excess	mmol/L	-9.0 ± 8.5 [-30.0 – 0.1] (23)	-19.1 ± 8.9 [-28.2 – -1.0] (25)	0.052	NS
Lactate	mmol/L	2.2 ± 1.2 [1.0 – 6.0] (19)	2.2 ± 2.0 [0.7 – 6.8] (19)	0.353	NS
Ammonia	µmol/L	934 ± 629 [170 – 2767] (20)	137 ± 104 [49 – 400] (19)	<0.001	**<0.001**
Haemoglobin	mmol/L	9.1 ± 1.7 [5.8 – 13.7] (22)	6.9 ± 1.0 [5.2 – 9.0] (25)	<0.001	**<0.001**
Thrombocytes	$*10^9$/L	292 ± 103 [6 – 358] (22)	321 _ 158 [98 – 892] (22)	0.027	NS
Leukocytes	$*10^9$/L	6.2 ± 4.8 [1.8 – 21.0] (23)	8.0 ± 7.4 [0.8 – 33.6] (25)	0.108	NS
Interventions					
Intensive care unit admission		*n* = 18; 62%	*n* = 15; 44%	0.208	NS
Mechanical ventilation		*n* = 13; 45%	*n* = 10; 29%	0.294	NS
Dialysis (any type) due to hyperammonemia		*n* = 11; 38%	*n* = 0; 0%	<0.001	**<0.001**

Early onset: presentation ≤28 days of life; Late onset: presentation >28 days of life; Min.: minimum value; Max.: maximum value; NS: not significant. Student's t-tests were performed for quantitative data and Fisher's exact tests were performed for qualitative data. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold.

Supplementary table 5. Adverse outcome due to the first symptomatic phase

	Propionic acidemia (*n* = 31)			**Methylmalonic acidemia (*n* = 45)**		**P-value**	**Bonferroni correction**
No AO	*n* = 14; 45%			*n* = 26; 58%		0.352	NS
Mild AO	*n* = 15; 48%			*n* = 15; 33%		0.235	NS
Severe AO	*n* = 1; 3%			*n* = 3; 7%		0.641	NS
Death due to AO	*n* = 1; 3%			*n* = 1; 2%		1.000	NS
	Early onset (n = 15)	***Late onset (n = 8)***	**P-value**	***Early onset (n = 14)***	***Late onset (n = 26)***	**P-value**	
No AO	*n* = 4; 27%	*n* = 6; 75%	**0.039**	*n* = 7; 50%	*n* = 17; 65%	0.500	NS; NS
Mild AO	*n* = 10; 67%	*n* = 1; 13%	**0.027**	*n* = 7; 50%	*n* = 5; 19%	0.071	NS; NS
Severe AO	*n* = 0; 0%	*n* = 1; 13%	0.348	*n* = 0; 0%	*n* = 3; 12%	0.539	NS; NS
Death due to AO	*n* = 1; 7%	*n* = 0; 0%	1.000	*n* = 0; 0%	*n* = 1; 4%	1.000	NS; NS
				Vitamin B12 unresponsiveness (n = 24)	***Vitamin B12 responsiveness (n = 21)***	**P-value**	
No AO				*n* = 12; 50%	*n* = 14; 67%	0.366	NS
Mild AO				*n* = 11; 46%	*n* = 4; 19%	0.068	NS
Severe AO				*n* = 1; 4%	*n* = 2; 10%	0.592	NS
Death due to AO				*n* = 0; 0%	*n* = 1; 5%	0.467	NS

AO: adverse outcome; Early onset: presentation ≤28 days of life; Late onset: presentation >28 days of life; NS: not significant. Statistical significance was determined by performing Fisher's exact tests. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant.

Supplementary table 6. Acute metabolic decompensations.

	Propionic acidemia (*n* = 31) *n* = ; median ± SD /PY [range]	**Methylmalonic acidemia (*n* = 45)** *n* = ; median ± SD /PY [range]	**P-value**	**Bonferroni correction**
Total	394; 0.7 ± 1.6 [0.0 – 7.3]	568; 0.4 ± 1.1 [0.0 – 4.7]	0.257	NS
Early onset	293; 1.2 ± 1.3 [0.0 – 4.2]	254; 0.9 ± 1.4 [0.0 – 4.7]	0.604	NS
Late onset	22; 0.2 ± 0.2 [0.0 – 0.7]	242; 0.2 ± 0.9 [0.0 – 3.5]	0.032	NS
Family testing	79; 0.8 ± 2.7 [0.0 – 7.3]	72; 0.6 ± 1.1 [0.0 – 2.8]	0.327	NS
Vitamin B12 unresponsive		504; 1.0 ± 1.1 [0.0 – 4.7]		
Vitamin B12 responsive		64; 0.1 ± 0.8 [0.0 – 3.5]	0.001	**0.001**
Age:				
<1 years	55; 1.0 ± 2.0 [0.0 – 7.0]	38; 0.0 ± 1.4 [0.0 – 5.0]	0.032	NS
1-3 years	136; 1.0 ± 2.0 [0.0 – 8.3]	156; 0.3 ± 1.7 [0.0 – 6.7]	0.485	NS
4-11 years	146; 0.3 ± 0.9 [0.0 – 3.5]	206; 0.1 ± 1.0 [0.0 – 5.0]	0.940	NS
12-17 years	40; 0.0 ± 0.5 [0.0 – 2.0]	85; 0.0 ± 0.8 [0.0 – 4.0]	0.483	NS
≥18 years	17; 0.0 ± 0.4 [0.0 – 1.8]	73; 0.0 ± 1.2 [0.0 – 9.9]	0.145	NS
Admission duration:				
1 – 3 days	110; 28%	207; 36%	0.006	**0.026**
4 – 7 days	143; 36%	164; 29%	0.017	NS
8 – 14 days	80; 20%	96; 17%	0.203	NS
≥ 15 days	51; 13%	45; 8%	0.012	**0.047**
NA	10; 3%	56; 10%		
ICU admission	23; 5.8%	18; 3.1%	0.051	NS
Triggers:				
Upper RTI	114; 29%	154; 27%	0.559	NS
Unknown	77; 20%	141; 25%	0.060	NS
Gastro-enteritis	75; 19%	111; 20%	0.868	NS
Feeding problems	16; 4%	50; 9%	0.004	**0.033**
Bacterial infection	24; 6%	32; 6%	0.781	NS
Constipation	39; 10%	4; 1%	<0.001	**<0.001**
Chronic instability	13; 3%	9; 2%	0.123	NS
Protein overload	4; 1%	4; 1%	0.723	NS
Other	16; 4%	11; 2%	0.072	NS
NA	16; 4%	52; 9%		
AMD frequency				
None	7; 23%	14; 31%	0.448	NS
Mild	2; 6%	2; 4%	1.000	NS
Moderate	1; 3%	3; 7%	0.641	NS
Severe	6; 19%	6; 13%	0.532	NS
Very severe	8; 26%	10; 22%	0.787	NS
Death	5; 16%	1; 2%	0.038	NS
NA	2; 6%	9; 20%		

AMD: Acute metabolic decompensation; early onset: presentation ≤28 days of life; late onset: presentation >28 days of life; RTI: respiratory tract infection; PY: patient year; NS: not significant; NA: not assessed. Student's t-tests were performed for quantitative data and Fisher's exact tests were performed for qualitative data. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold.

Supplementary table 7. Complications with potential mitochondrial etiology.

Mitochondrial Pathophysiology[a]		Propionic acidemia (*n* = 31)		Methylmalonic acidemia (*n* = 45)		P-value	Bonferroni correction
		This cohort	*Lit.*	*This cohort*	*Lit.*		
Mitochondrial complications during first pres.							
Basal ganglia hyperintensities	Probably	*n* = 10; 32%	22%	*n* = 8; 18%	28%	0.175	NS
White matter lesions		*n* = 6; 19%	39%	*n* = 8; 18%	32%	1.000	NS
Complications related to first presentation							
Cerebral atrophy		*n* = 13; 42%		*n* = 11; 24%		0.135	NS
Movement disorders		*n* = 10; 32%	16%	*n* = 14; 31%	32%	1.000	NS
Psychomotor retardation		*n* = 19; 61%	49%	*n* = 24; 53%	53%	0.638	NS
Cognitive dysfunction		*n* = 20; 65%	69%	*n* = 19; 42%	58%	0.066	NS
Group 1: IQ >90 or regular education		*n* = 7; 23%		*n* = 17; 38%		0.212	NS
Group 2: IQ 60-90 or special education		*n* = 7; 23%		*n* = 13; 29%		0.604	NS
Group 3: IQ <60 or no education		*n* = 13; 42%		*n* = 6; 13%		0.007	**0.021**
NA		*n* = 4; 13%		*n* = 9; 20%			
Mitochondrial complications with acute onset							
Hepatomegaly and/or hyperechogenic liver	Probably	*n* = 11; 35% 3.3 y (0.1 – 57.1)	78%	*n* = 14; 31% 0.4 y (0.0 – 9.9)	33%	0.805	NS
Epilepsy	Probably	*n* = 7; 23% 0.0 y (0.0 – 6.3)	23%	*n* = 2; 4% 2.1 y (0.0 – 4.2)	13%	0.027	NS
Cardiomyopathy	Probably	*n* = 7; 23% 8.5 y (7.5 – 56.3)	14%	*n* = 1; 2% 23.3 y	5%	0.007	NS
Optic atrophy	Probably	*n* = 4; 13% 15.9 y (11.8 – 16.7)	5%	*n* = 4; 9% 20.8 y (12.6 – 26.9)	6%	0.709	NS
Pancreatitis	Probably	*n* = 1; 3% 20.8 y	5%	*n* = 2; 4% 19.1 y (9.5 – 28.7)	4%	1.000	NS
Renal failure	Possibly	*n* = 1; 3% 21.4 y	1%	*n* = 20; 44% 8.9 y (1.2 – 31.3)	29%	<0.001	**<0.001**
Sensorineural hearing loss	Possibly	*n* = 6; 19% 6.6 y (2.0 – 8.5)	4%	*n* = 3; 7% 4.8 y (2.5 – 9.8)	2%	0.434	NS
Acute psychosis	Possibly	*n* = 3; 10% 17.2 y (16.8 – 23.5)		*n* = 0; 0%		0.064	NS
Stroke-like episodes	Possibly	*n* = 2; 7% 13.4 y (9.0 – 17.9)	14%	*n* = 0; 0%	17%	0.163	NS
Prolonged QTc interval	Unknown	*n* = 7; 23% 8.5 y (0.0 – 38.3)	31%	*n* = 4; 9% 14.0 y (3.6 – 28.4)	2%	0.300	NS
Premature ovarian insufficiency	Unknown	*n* = 0; 0%		*n* = 1; 2% 19.1 y		1.000	NS

Mitochondrial complications with chronic onset							
Exercise intolerance	Probably	*n* = 15; 48%		*n* = 10; 22%		0.025	NS
Autism	Probably	*n* = 2; 6%	9%	*n* = 4; 9%		1.000	NS
Feeding problems	Possibly	*n* = 18; 58%		*n* = 22; 49%		0.488	NS
Muscular hypotonia	Possibly	*n* = 13; 42%	45%	*n* = 21; 47%		0.815	NS
Constipation	Unknown	*n* = 14; 45%		*n* = 9; 20%		0.024	NS
Attention deficit hyperactive disorder		*n* = 1; 3%	15%	*n* = 1; 2%		1.000	NS
Mitochondrial complications, intermittent occurr.							
Anemia	Possibly	*n* = 21; 68%	51%	*n* = 28; 62%		0.807	NS
Leukopenia	Possibly	*n* = 19; 61%	31%	*n* = 16; 36%		0.036	NS
Thrombocytopenia	Possibly	*n* = 19; 61%	28%	*n* = 23; 51%		0.483	NS
Pancytopenia	Possibly	*n* = 13; 42%	19%	*n* = 9; 20%	0%	0.044	NS
Mitochondrial complications							
None		*n* = 3; 10%		*n* = 3; 7%		0.683	NS
Mild		*n* = 8; 26%		*n* = 22; 49%		0.057	NS
Moderate		*n* = 3; 9%		*n* = 10; 22%		0.219	NS
Severe		*n* = 5; 16%		*n* = 7; 16%		1.000	NS
Very severe		*n* = 11; 35%		*n* = 3; 7%		0.002	**0.013**
Death		*n* = 1; 3%		*n* = 0; 0%		0.408	NS

a: Likelihood of mitochondrial pathophysiology[2]; Lit: percentage reported in literature[2]; NS: not significant; NA: not assessed. Statistical significance was determined by performing Fisher's exact tests. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold.

Supplementary table 8. Prevalence of treatment-related and miscellaneous complications.

	Propionic acidemia (*n* = 31)	Methylmalonic acidemia (*n* = 45)	P-value	Bonferroni correction
Treatment-related complications				
Reduced bone mineral density	*n* = 9; 29%	*n* = 18; 40%	0.465	NS
Growth retardation/short stature	*n* = 8; 26%	*n* = 13; 29%	0.801	NS
Obesity	*n* = 10; 32%	*n* = 6; 13%	0.084	NS
Treatment-related complications				
None	*n* = 12; 39%	*n* = 21; 47%	0.638	NS
Mild	*n* = 13; 42%	*n* = 15; 33%	0.477	NS
Severe	*n* = 4; 13%	*n* = 5; 11%	1.000	NS
Very severe	*n* = 2; 6%	*n* = 4; 9%	1.000	NS
Miscellaneous complications				
Pes planovalgus	*n* = 9; 29%	*n* = 13; 29%	1.000	NS
Port-a-cath infections	*n* = 3; 10%	*n* = 2; 4%	0.393	NS
Enamel defects	*n* = 1; 3%	*n* = 3; 7%	0.641	NS
Urolithiasis	*n* = 1; 3%	*n* = 1; 2%	1.000	NS
Gout	*n* = 0; 0%	*n* = 2; 4%	0.511	NS

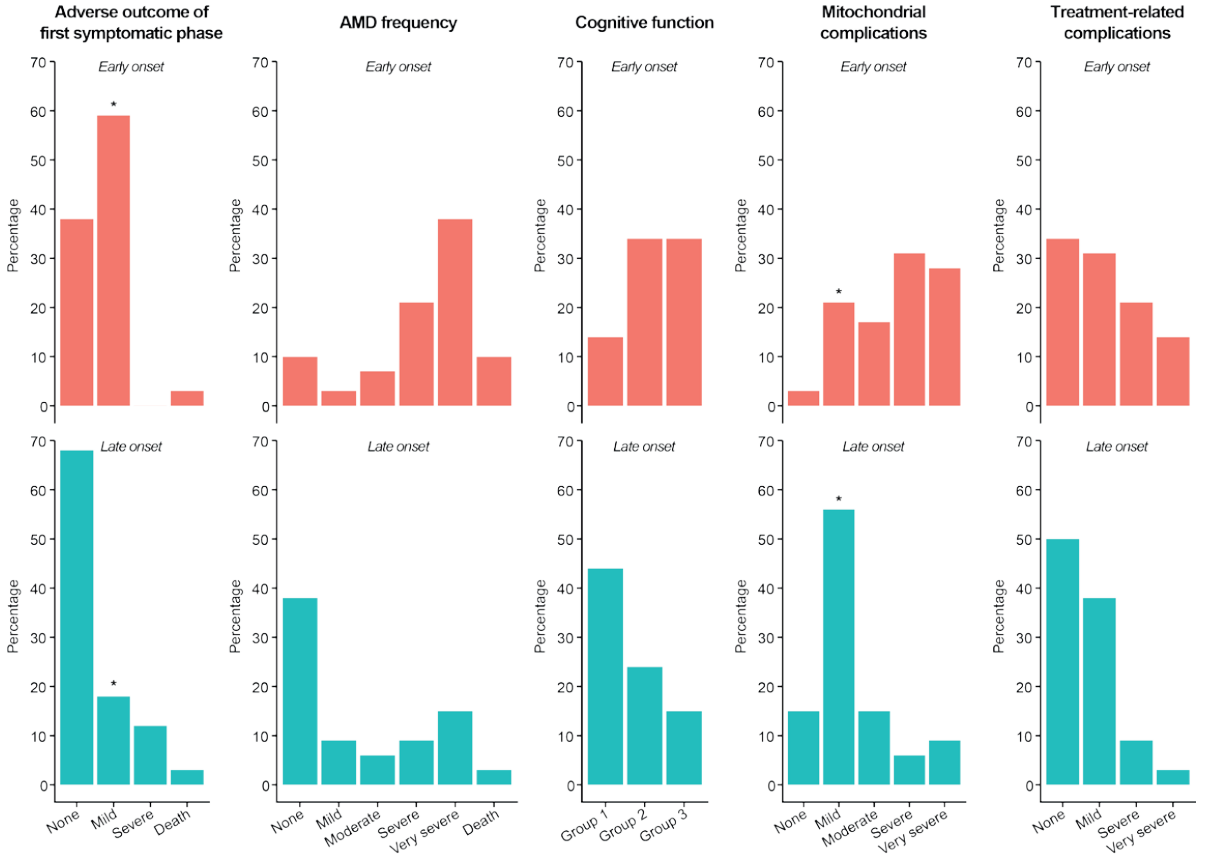
NS: not significant. Statistical significance was determined by performing Fisher's exact tests. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold.

Supplementary table 9. Minimum requirements for follow-up of the post-NBS cohort.

	Items to record	Diagnostics to perform
Patient characteristics		
Patient	Day of birth Sex	
Family	Ancestry Consanguinity	
Mutation	g. / c. / p. coding sequence	Genetic testing
Vitamin B12 responsiveness	Type enzymatic assay Results enzymatic assay	Enzymatic assay B12 responsiveness
PCC activity	Type enzymatic assay Results enzymatic assay	Enzymatic assay PCC activity
Death	Day of death Cause of death	
Follow-up	Age at last follow-up	
Adverse outcome of first sympt. phase		
First presentation	Symptomatic/asymptomatic Day of first symptoms Day of diagnosis	
Adverse outcome first presentation	Day of brain MRI Brain MRI results	Brain MRI at set times
	Movement disorder	Consult neurologist
AMD frequency		
Number of AMD	Day of admission Day of release Reason of admission	
Cognitive function		
Cognition	Day of neuropsychological tests Neuropsychological test type Neuropsychological test results; IQ	Neuropsychological tests at set times
Education	School career Type of employment	
Mitochondrial complications		
Hepatomegaly	Day of diagnostic study	Liver ultrasound at set times
Epilepsy	Results of diagnostic study	EEG at set times
Cardiomyopathy	Presence complication yes/no	Cardiac ultrasound at set times
Prolonged QTc interval		ECG at set times
Optic atrophy		Consult ophthalmologist at set times
Renal failure		Urine kidney function biochemistry at set times
Pancreatitis		Complete blood count at set times
Sensorineural hearing loss Acute psychosis Stroke-like episodes Premature ovarian insufficiency Exercise intolerance Muscular hypotonia Feeding problems Constipation		On indication: consult ENT doctor, gastro-enterologist, neurologist, gynecologist, psychiatrist, physical therapist

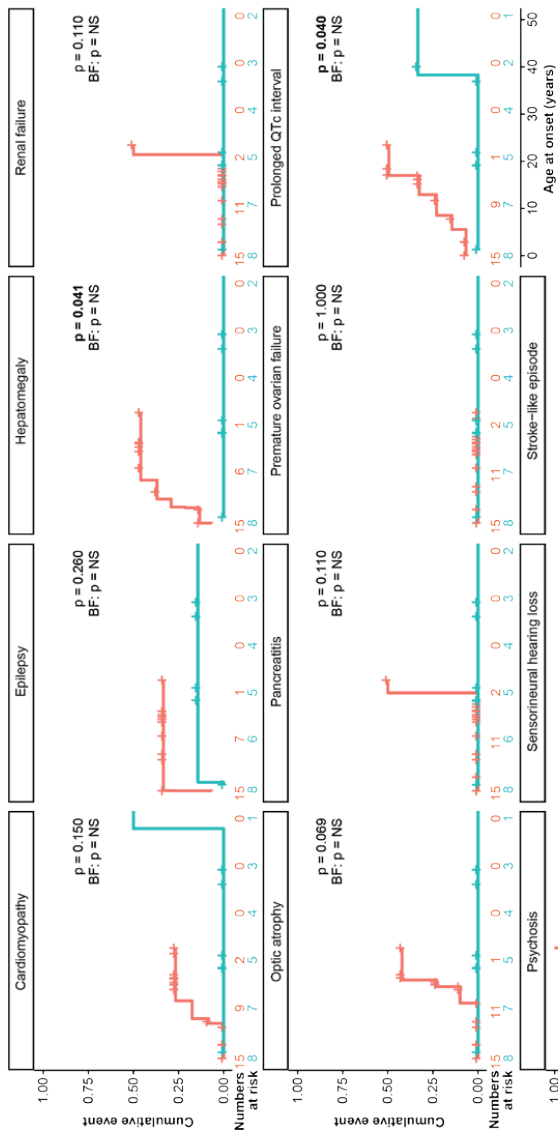
Autism Attention deficit hyperactive disorder Anemia Leukopenia Thrombocytopenia Pancytopenia		
Treatment-related complications		
Bone mineral density Growth retardation Obesity	Day of diagnostic study Results of diagnostic study Presence complication yes/no Visit date each visit Weight and length each visit	DEXA-scan at set times

g./c./p. coding sequence: genetic change in the DNA coding sequence and resulting change in the protein coding sequence. Adverse outcome of first sympt. phase: Adverse outcome of the first symptomatic phase. DEXA: dual-energy X-ray absorptiometry; ECG: electrocardiogram; EEG: electroencephalogram; ENT: ear-nose-throat; MRI: magnetic resonance imaging; PCC: propionyl-CoA carboxylase; IQ: intelligence quotient.



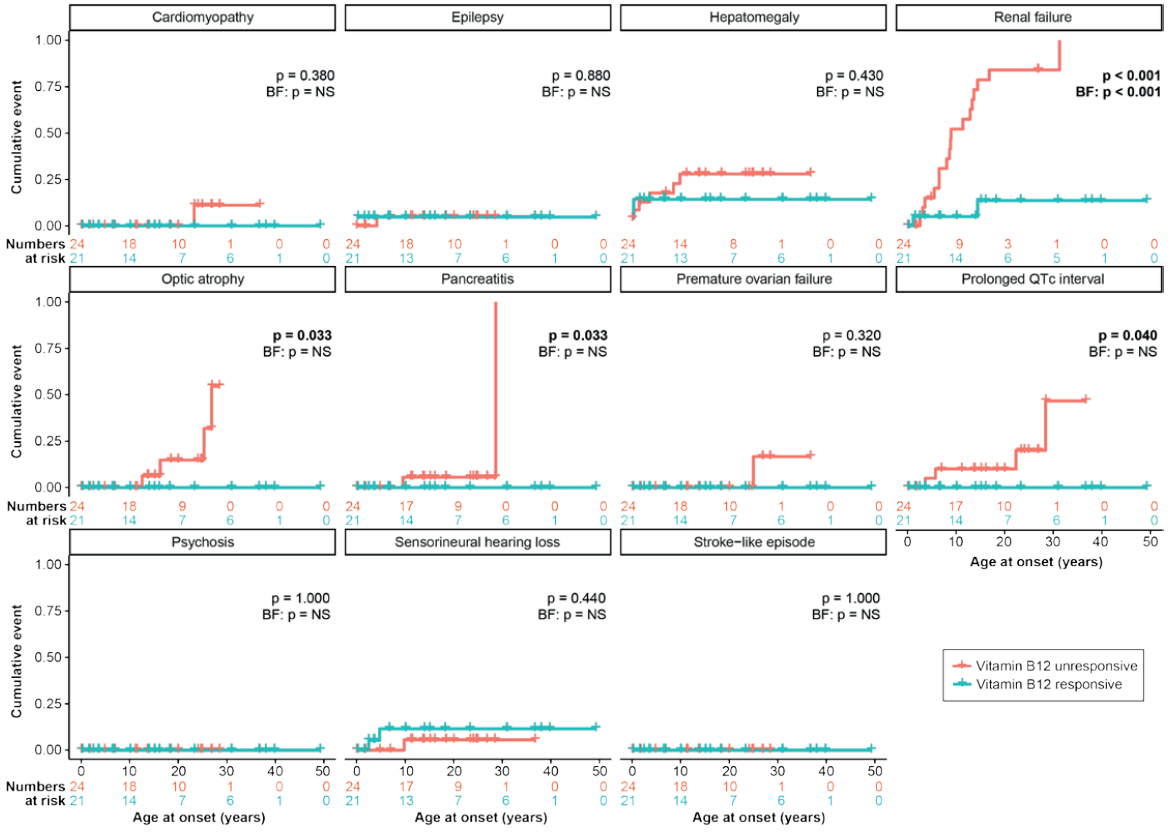
Supplementary figure 1. Grouped outcome parameters according to presentation type.

Visual representation of Table 2. Categories of the outcome parameters are depicted on the x-axis, percentages are depicted on the y-axis. Early onset (presentation ≤28 days of life) is depicted in orange, late onset (presentation >28 days of life) is depicted in blue. Statistical significance was determined by fisher's exact tests. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted with bold asterisks, as in Table 2.

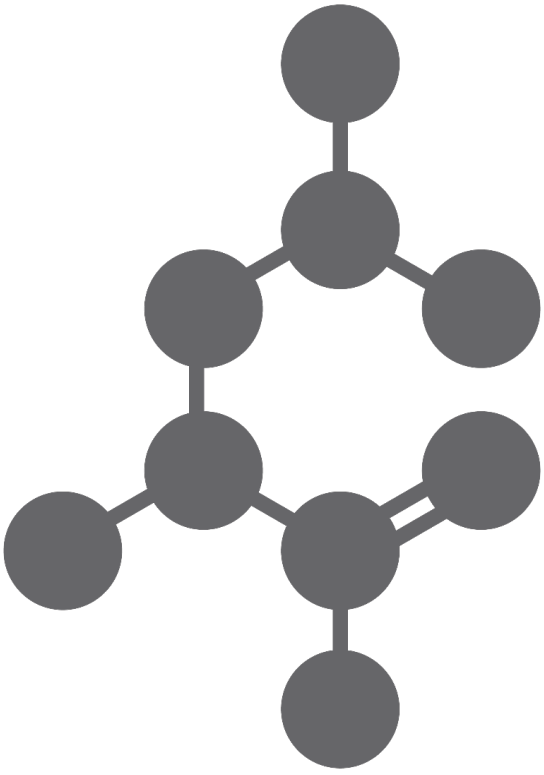


Supplementary figure 2. Early onset presentation in PA tends to be an independent predictor for four mitochondrial complications with acute onset.

Kaplan-Meier plots wherein the y-axis depicts the cumulative percentage and the x-axis depicts patient age in years. The panels demonstrate the different mitochondrial complications with acute onset, for patients with late onset presentation in blue versus patients with early onset presentation in orange. Numbers at risk, indicating the number of patients at risk for a certain complication are depicted below the panels, in corresponding colors. Early onset: presentation ≤28 days of life; Late onset: presentation >28 days of life. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold. BF: Bonferroni, NS: not significant.



Supplementary figure 3. Vitamin B12 unresponsiveness in MMA tends to be an independent predictor for three mitochondrial complications with acute onset and is a significant predictor for the occurrence of renal failure.
Kaplan-Meier plots wherein the y-axis depicts the cumulative percentage and the x-axis depicts patient age in years. The panels demonstrate the different mitochondrial complications with acute onset, for vitamin B12 responsive patients in blue versus vitamin B12 unresponsive patients in orange. Numbers at risk, indicating the number of patients at risk for a certain complication are depicted below the panels, in corresponding colors. All p-values were adjusted according to the Bonferroni method. Adjusted p-values < 0.05 were considered statistically significant and are depicted in bold. BF: Bonferroni, NS: not significant.



LIVER AND/OR KIDNEY TRANSPLANTATION IN AOA PATIENTS

Neurotoxicity including PRES after initiation of calcineurin inhibitors in transplanted methylmalonic acidemia patients: two case reports and review of the literature.

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Submitted

7.2

Abstract

Introduction: New neurological symptoms in methylmalonic acidemia (MMA) patients after liver and/or kidney transplantation (LKT) are often described as metabolic stroke-like-events. Since calcineurin inhibitors (CNIs) are a well-known cause of new neurological symptoms in non-MMA transplanted patients, we investigated the incidence of CNI-induced neurotoxicity including posterior reversible encephalopathy syndrome (PRES) in post-transplanted MMA patients.

Methods: We report the 2 MMA patients treated with LKT in our center. Additionally, we performed a systematic review of case reports/series of post-transplanted MMA patients and determined if CNI-induced neurotoxicity/PRES was a likely cause of new neurological symptoms. Definite CNI-induced neurotoxicity was defined as new neurological symptoms during CNI treatment with symptom improvement after CNI dose reduction/discontinuation. PRES was defined as CNI-induced neurotoxicity with signs of vasogenic edema on brain MRI-scan post-transplantation.

Results: Our 2 MMA patients both developed CNI-induced neurotoxicity, 1 had PRES. In literature 230 transplanted MMA patients were identified. Neurological follow-up was reported in 54 of them, of which 24 were excluded from analysis since no anti-rejection medication was reported. Thirty patients, all using CNI, were included. Sixteen patients (53%) had no new neurological symptoms post-transplantation and five patients (17%) had definite CNI neurotoxicity of whom 2 had PRES. Including our cases this results in a pooled incidence of 22% (7/32) definite CNI neurotoxicity and 9% PRES (3/32) in post-transplanted MMA patients on CNI.

Conclusion: In MMA post-transplanted patients with new neurological symptoms CNI-induced neurotoxicity/PRES should be considered. Early recognition of CNI-induced neurotoxicity is essential to initiate dose reduction/discontinuation of CNI in order to minimize persistent neurologic damage and improve outcome.

1 | Introduction

Methylmalonic acidemia (MMA) is a severe rare inborn error of metabolism, belonging to the organic acidemias. MMA leads to increased levels of methylmalonic acid (mma). Isolated MMA is caused by complete (*mut*^{0}) or partial (*mut*$^-$) deficiency of the mitochondrial enzyme methylmalonyl-CoA mutase (MUT) (OMIM #251000) or by deficient synthesis of the MUT-cofactor adenosylcobalamin (CblA (OMIM #251100) or CblB (OMIM #251110)) [1]. While survival of MMA patients has greatly improved over the past decades with conventional treatment strategies [2, 3], patients continue to develop serious long-term complications [4], including renal insufficiency and neurological complications such as developmental delay, seizures and metabolic stroke [5]. Furthermore patients have an impaired quality of life [6].

Since the prognosis of MMA patients is often poor, liver and/or kidney transplantation is performed with increased frequency [7, 8]. Although the liver is the main site of MUT enzyme expression the enzyme is expressed in other tissues as well [9], including the kidneys and in lesser extent the muscles and brain [10]. Hence liver and/or kidney transplantation does not fully restore MUT enzyme activity. The outcome of transplantations in MMA patients varies and there are multiple reports of patients who developed new neurological complications after transplantation [11, 12]. Concerns about new neurological complications after transplantation is well described and it is mentioned in a recent guideline on organic acidurias ([5]). The new neurological complications after transplantation can be due to deficient MUT activity in non-transplanted tissues leading to high mma levels in cerebrospinal fluid [13, 14] and cerebral tissues or to metabolic encephalopathy during decompensations.

However, new neurological complications are also a disturbing and relatively common phenomena in non-MMA patients after organ transplantation (occurring in 15%-40% of patients) [15-17]. These neurological complications can be caused by a variety of factors including immunosuppressive medication (especially calcineurin inhibitors (CNI), such as tacrolimus and cyclosporine) [16]. CNI-induced neurotoxicity in non-MMA organ transplanted patients can present with a variety of symptoms such as confusion, tremor, seizures, cerebral hemorrhage, ischemic stroke and posterior reversible encephalopathy syndrome (PRES) [17-20]. In CNI-induced PRES, the brain magnetic resonance imaging (MRI)-scan typically shows bilateral areas of vasogenic edema, which may be located in the cortex and/or subcortical

white matter of brain areas; supplied by posterior circulation. However an atypical distribution pattern is not infrequently observed, e.g. involving the basal ganglia and/or the brain stem [18], with or without atypical imaging findings (contrast enhancement, hemorrhage or restricted diffusion) [20].

Recently, two adult MMA patients received combined liver/kidney transplantation in our center. They were the first transplanted MMA patients in our center, and both patients had new neurological symptoms within months post transplantation, which we attributed to CNI-induced neurotoxicity. The aim of this study was to investigate the occurrence of CNI-induced neurotoxicity in liver and/or kidney transplanted MMA patients. Therefore we describe the two transplanted MMA patients in our center and performed a systematic review of previously published case reports and case series of MMA patients following liver and/or kidney transplantation. We retrospectively reviewed if neurological symptoms could be attributed to CNI-induced neurotoxicity. Furthermore, we calculated the pooled incidence of CNI-induced neurotoxicity, including PRES, in MMA patients in our center and from literature.

2 | Methods

To identify all published case reports and case series of transplanted MMA patients, we performed a systematic search of MEDLINE and EMBASE databases on the 11th of September 2018. Search terms were 'mma OR (methylmalonic AND acidemia) OR (methylmalonic AND aciduria) OR (methylmalonic AND acidaemia) OR mcm OR (methylmalonyl AND coa AND mutase) OR mut OR (mcm AND deficiency) OR (acidemia AND methylmalonic) OR (cblb AND type) OR (cblb AND disease) OR (cblb AND disorder) OR (cbla AND type) OR (cbla AND disease) OR (cbla AND disorders) OR mut- OR mut0 OR mmaa OR mmab AND transplantation OR transplant OR grafting OR graft OR implantation OR implant AND liver OR hepatic OR kidney OR renal OR combined OR (liver AND kidney) OR clkt' in Embase and Pubmed. Exclusion criteria were another disease than MMA and in vitro or animal studies. Duplicate patients identified by the same author(s) and same age at transplantation (when reported) and/or mutation (when reported) were excluded. Informed consent of the two Erasmus MC patients was obtained.

2.1 | Definitions

We defined the condition of 'definite CNI-induced neurotoxicity' as new neurological symptoms that started while the patient was using CNI, as anti-rejection medication, within the first year after transplantation and improvement following dose reduction or discontinuation. We attributed new neurological symptoms after transplantation to 'probable CNI induced neurotoxicity' as 1) patients with new seizures, unlikely due to another cause than CNI, within one year after transplantation (in case time period was mentioned) without reported anti-rejection medication or 2) newly acquired symptoms, unlikely due to another cause than CNI, within one year after transplantation while on CNI with unclear outcome reported of neurological symptoms. We attributed 'CNI-induced PRES' to 'definite CNI-induced neurotoxicity' in combination with 1) brain MRI-scan abnormalities, with typical imaging findings (signs of vasogenic edema) with a typical (in parieto-occipital and/or posterior frontal cortex and/or subcortical white matter) or an atypical distribution (in other brain areas such as brain stem, basal ganglia, subcortical or cortical frontal regions without a posterior predominance), with or without atypical imaging findings (such as contrast enhancement, hemorrhage and or restricted diffusion on brain MRI-scan) and either 2a) improvement of brain MRI-scan findings on follow-up or, in case of unavailability of follow-up MRI 2b) return to baseline neurological status after dose reduction or discontinuation of CNI [15, 20, 21].

2.2 | Data analysis from the literature

Characteristics of all described outcome parameters of each reported case were derived from the reports that were found via the literature search. Three reviewers (F.M., Ma.Wa. and Mo.Wi.) independently judged whether or not the cases were described in enough detail to conclude that they had 1) generally reported follow-up after solid organ transplantation, 2) reported neurological follow-up. Subsequently, the same reviewers independently decided in which group the patients with new neurological symptoms should be included, i.e. 1) patients without reported post-transplantation anti-rejection medication, 2) patients with reported anti-rejection medication, either with or without neurological symptoms. Finally the reviewers retrospectivity attributed new neurological symptoms to 1) 'definite CNI-induced

neurotoxicity', subdividing them into patients with and without PRES 2) 'probable CNI-induced neurotoxicity, 3) neurological symptoms due to another cause (such as stroke-like event). Thereafter consensus was made on those cases that were non-conclusive.

2.3 | Analysis of FGF-21 concentrations in the two cases from the Erasmus MC

We measured serum levels of FGF-21 levels in the two patients transplanted in our center on several time points before and after transplantation and during neurological events. All were measured using a commercial ELISA kit (Millipore) according to local protocol, as reported in Molema et al [22].

2.4 | Analysis of MRI findings in the two cases from our center

The MRI-scans, existing of T1-weighted, T2-weighted and diffusion weighted imaging (DWI) before, and T1-weighted and 3D FLAIR imaging after gadolinium injection, were performed in a clinical setting. The initial scan of case 1 additionally included susceptibility weighted imaging and contrast enhanced MR angiography. The neuroradiologist (AE) from the Erasmus MC performed a second reading of the MRI-scans, taking into account the original radiological reports.

2.5 | Statistical analysis

Descriptive parameters (frequencies, median and range) were used as outcome measurements in this study. The pooled incidence of CNI-induced neurotoxicity, including PRES, was calculated by combining the patients from literature and the patients from our center.

3 | Results

3.1 | Case reports from our center

Case 1:

A 29 year old female patient was diagnosed with MMA at the age of 4.5 months while presenting with coma, vomiting and hepatomegaly. The patient was non-vitamin b12 responsive and the MMA was caused by a homozygous frameshift mutation in the MUT gene (c.1311_1312insA) (table 1). At the age of 28 years she had been admitted to the hospital 58 times for metabolic derangements. She had a mild intellectual disability; she worked as a shop-assistant. She had developed visual loss at 12 years of age and progressive renal insufficiency. At age 28 years she required hemodialysis and she opted for a combined LKT. There were no peri-operative complications. She was placed on an immunosuppressive regime of tacrolimus, prednisone and mycophenolate mofetil (MMF) and her diet was liberated to a non-restricted diet. Of the previous medication only carnitine and vitamin b12 supplementation were continued. She recovered well without any metabolic disturbance.

Approximately 2.5 months after the transplantation, she started feeling unwell without objective abnormalities during physical and laboratory examination. After two weeks (postoperative day (POD) 83) she presented with headache, ataxia and bradyphrenia. An MMA related metabolic stroke was initially considered, although no metabolic decompensation was present (according to laboratory characteristics), and a strict emergency regime was prescribed. Her brain MRI-scan (POD83) showed abnormalities: 1) symmetrical T2 hyperintense laesions in basal ganglia (figure 1a1), mammillary bodies (figure 1a2), pons and cerebellum (figure 1a3), 2) swelling and faint contrast enhancement in basal ganglia (figure 1a1), swelling and avid contrast enhancement in the mammillary bodies (figure 1a2) and 3) high signal on DWI with intermediate ADC of the basal ganglia (figure 1a1), no diffusion restriction in the mammillary bodies (figure 1a2), and diffusion restriction of some of the cerebellar lesions (figure 1a3) (supplementary table 1). Due to movement artefacts, no reliable assessment of contrast enhancement of the cerebellar abnormalities was possible (figure 1a3). These findings can be caused by a metabolic (MMA induced) stroke-like episode but also by PRES. The involvement of the mammillary bodies is atypical for both etiologies. Furthermore,

a lentiform fork sign was observed, suggestive of metabolic acidosis, which was biochemically not confirmed. Also calcifications in anterior limb of internal capsule (figure 1a4) were observed. At POD85 mma was still very low and tacrolimus levels were within the normal non-toxic range (table 1). The patient did not improve with the emergency regime and a tacrolimus induced PRES was suggested. The tacrolimus was discontinued at POD86 and replaced by everolimus. Within a week the clinical picture of the patient clearly improved, apart from a severe depression (POD 93), which was successfully treated with medication and psychotherapy.

Three months later a new brain MRI-scan (POD193) showed decreased signal abnormalities, however with tissue loss in basal ganglia, mammilary bodies and diffuse atrophy in SCA territory and focal loss of tissue at the locations of the lesions in the –PICA and AICA territories of the cerebellum. At 14 months follow up the ataxia has greatly improved but mild bradyphrenia, orophacial dyskinesia (which is thought to be caused by citolapram) and a mild left sided hemiparesis is still present. In conclusion: because the time of onset of the new neurological symptoms after transplantation, the abnormalities on MRI scan and the evident improvement of symptoms after discontinuation of tacrolimus this patient was diagnosed with a CNI-induced PRES.

Laboratory characteristics: mma and FGF-21.

Plasma mma values before transplantation ranged between 3000 -11700 µmol/L and after transplantation rapidly declined to 200-300 µmol/L on day 4 after transplantation (figure 2). Although FGF-21 values, measured retrospectively before transplantation, were very high (>4000 pmol/ml), they rapidly decreased after transplantation (<700pmol/ml). At POD85 when presenting with new neurological symptoms mma was still very low, but there was a significant rise in FGF-21 plasma levels (retrospectively) (figure 2). Cerebrospinal fluid (CSF) mma levels (728 µmol/l), measured once during the work-up of the neurological symptoms, were higher than in plasma with a CSF/plasma mma ratio of 2.7, conform reported in literature [13, 14]. FGF-21 normalized when she clinically recovered.

Table 1. Patient characteristics of patients with CNI-induced neurotoxicity.

Case	Case 1	Case 2	Giusanni	Niemi	Vernon	Mc Guire	Nagarajan
Year publication, gender	2019, f	2019, f	2016, m	2015, m	2014, f	2008, m	2005, m
Age onset, age at diagnosis	5mo, 5mo	12days, un	un, <1y	both un	3mo, 9mo	<3mo, 3mo	1 week, 1 week
Genotype	Mut0 c.1311_1312insA, p.Val438Serfs*3 *non-vitamin b12 responsive*	Mut0 c.2078delG, p.G693Dfs*12 *non-vitamin b12 responsive*	un	un	Mut0 c.2053dupCTC p.685insL *non-vitamin b12 responsive*	Mut0	Mut0
Neurologic complications pre-Tx	None	None	un	un	Mild choreoathesosis due to bilateral globus pallidus infarction	Aphasic and difficulty ambulating due to weakness and tremors	Deterioration with dystonia, muscular weakness and wheelchair bound
Other complications pre-Tx	Impaired vision due to optic nerve infarction, age 12y ESRD*, age 18y, she required hemodialysis	Near blind from sudden onset optical atrophy, age 17y Chronic renal insufficiency age 10y, stage IV age 18y	un	un	Acute bilateral opticus neuropathy Chronic kidney disease**	Renal insufficiency, frequent metabolic decompensations	Pancreatitis
Development pre-Tx	Normal: WAIS-IV performed at age 27y: normal with mild expressive language problems	Normal	un	un	Normal	un	Cognitive development delay, decreased motor skills

Age Tx	28y	19y	6y	un	28y	5y	21y
Duration follow-up	14mo	8mo	un	un	un	10mo	1y6mo
Tx	Combined LKT	Combined LKT (lost renal Tx)	Combined LKT	Liver	Combined LKT	Combined LKT	Combined LKT
Medication after Tx	Tacrolimus, MMF, prednisone	Tacrolimus, MMF, prednisone	Cyclosporine, prednisone	Tacrolimus, prednisone, azathioprine	MMF, prednisone, basiliximab, POD 6 tacrolimus	Tacrolimus and steroids	Tacrolimus, sirolimus, prednisone
Start neurological symptoms post-Tx	3mo	22 days	10 days	12 days	28 days and 48 days	Weeks	un
Neurological symptoms	Bradyphrenia, severe ataxia, behavioral changes	Seizures	Seizures	Seizures	Seizures	Altered mental status, aphasia, hallucinations, seizures, tremor	Altered mental status, tremors
Tacrolimus level during neurological symptoms (ng/mL)	10.7	3.1 - 5.5 ***	un	high	6.7	<5	5-7
Mma levels during neurological symptoms (µmol/l)	272 in plasma, 728 in the cerebrospinal fluid	268 in plasma	un	<343 in plasma	un	<500 in plasma	<500 in plasma
Final consensus diagnosis	CNI-induced PRES	Definite CNI-induced neurotoxicity	CNI-induced PRES	Definite CNI-induced neurotoxicity	CNI-induced PRES	Definite CNI-induced neurotoxicity	Definite CNI-induced neurotoxicity

Abbrevations. f=female; m=male; un = unavailable; Tx = transplantation; y=year, mo=months; combined LKT = combined liver and kidney transplantation; ESRD= end stage renal disease; * despite fluid up to 7L/d via PEG; ** secondary hyperparathyroidism, hypothyroidism; *** deliberately lower in setting of combination therapy with MMF due to renal failure.

Case 2 (previously reported in Molema et al [22] as case 12):

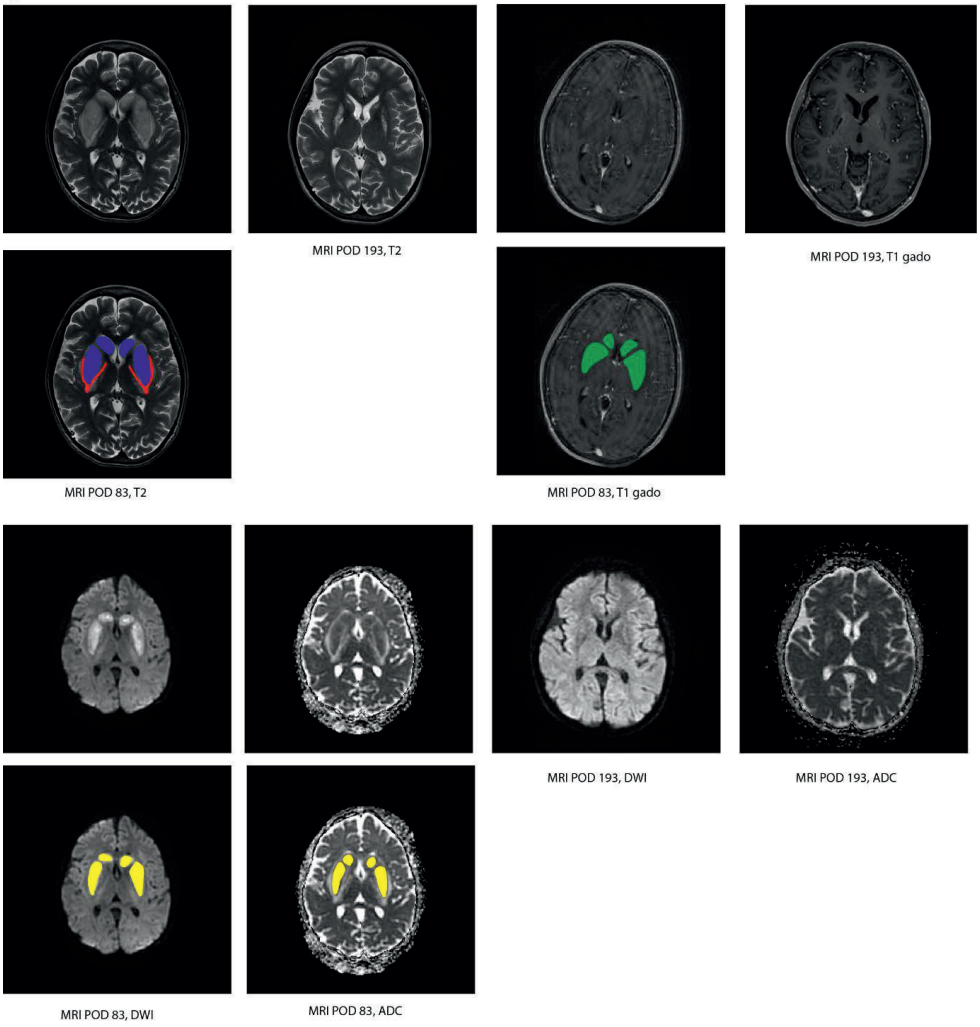
A 19 year old female was diagnosed with early onset MMA (at the age of 12 days) presenting with vomiting, weight loss and an ammonia level of 172 μmol/L. The MMA was caused by a homozygous frameshift mutation in the MUT gene (c.2078delG), and was non vitb12 response (table 1). At the age of 19 years the patient had been hospitalized a total of 22 times because of metabolic decompensations. At 16 years of age she developed rapid visual loss due to optical atrophy and a progressive renal insufficiency, stage 4 and had to leave school (higher secondary education). Because her clinical condition rapidly deteriorated she received a combined LKT at the age of 19 years and 2 months. Conform case 1; she was placed on an immunosuppressive regime of tacrolimus, prednisone and MMF. Whilst the transplantation itself was uncomplicated the direct postoperative period was complicated by primary kidney graft dysfunction caused by an arterial thrombosis in the anastomosis, which despite a thrombectomy resulted in graft kidney loss, which was removed at POD 2. At POD 7 the patient developed a life threatening hemorrhagic shock caused by acute gastrointestinal bleeding (Hb of 2.5 mmol/L) a complication of reflux esophagitis, LA grade C. She recovered well with proton pump inhibitors without neurological complications and the patient received 1 g/kg natural protein per day. On POD 31 she presented with a tonic clonic seizure, without signs of metabolic decompensation (according to laboratory characteristics). Triggered by the experience with case 1, CNI-induced neurotoxicity was immediately suspected even though tacrolimus levels were within normal non-toxic range and tacrolimus was switched to everolimus on POD31. The brain MRI-scan performed at POD 32 (supplementary figure 2) showed no signs of PRES nor other signal abnormalities. Coincidentally, a hypoplasia of the vermis cerebelli was found with enlarged fourth ventricle and mildly enlarged supratentorial ventricles. Because of recurrent seizures in the first week after her initial seizure, treatment with levetiracetam was started with good result and no persisting neurological symptoms. However the patient developed a status epilepticus on POD49, shortly after the everolimus dose was increased, thought to be caused by supra-therapeutically levetiracetam levels (42 mg/L) (in combination with use of everolimus). She was treated with Diphantoine and midazolam, levetiracetam dose was reduced and since everolimus could also be causative everolimus was temporary discontinued. One more episode of three seizures on occurred on POD 68, everolimus was again briefly discontinued. Currently the patient has a follow-up of 8 months

and she is functioning better than before transplantation (more energy and less nausea) with no lasting new neurological symptoms. She is now treated with everolimus, prednisolone, MMF, levetiracetam (which we plan to discontinue soon), levocarnitine and idebenon. In conclusion: because the time of onset of the new severe epilepsy after transplantation, which resolved after discontinuation of tacrolimus this patient was diagnosed with a CNI induced neurotoxicity.

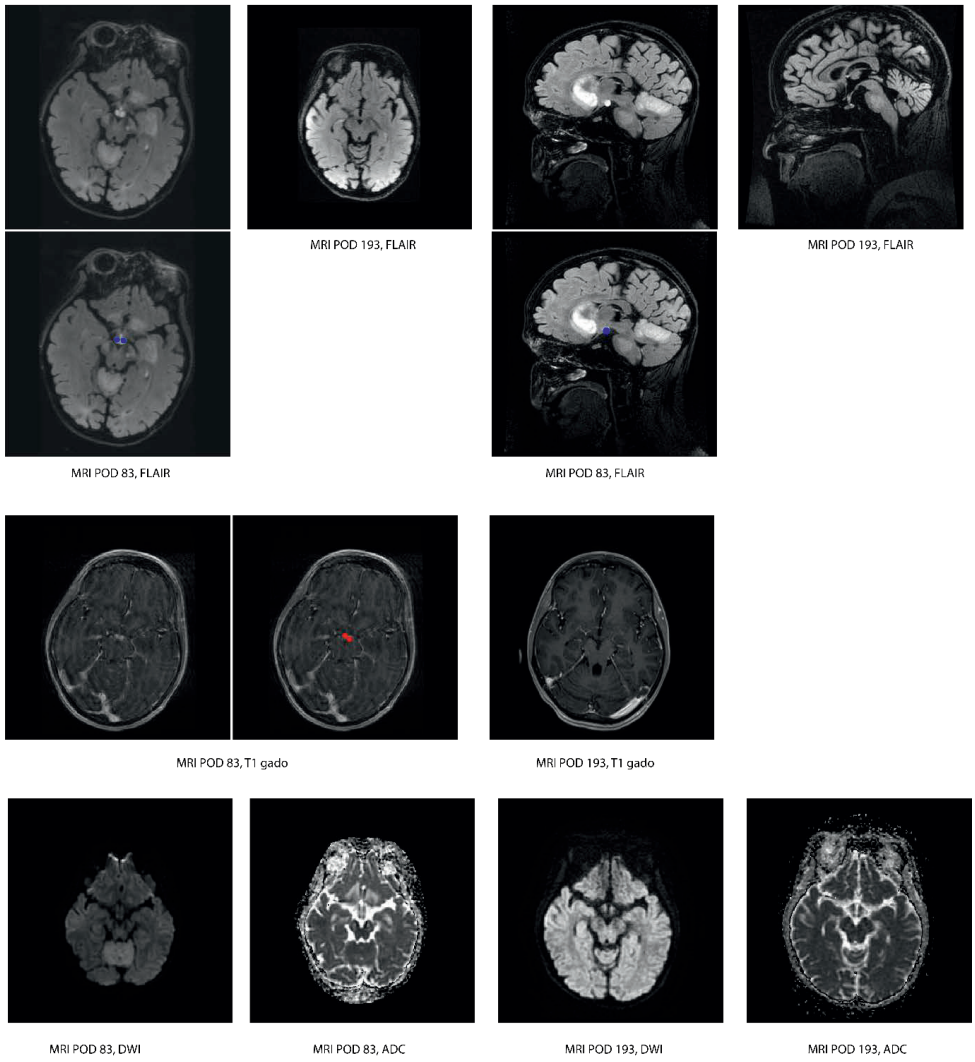
Laboratory characteristics: mma and FGF-21.

A significant drop in plasma mma and FGF-21 after transplantation was seen in both parameters (figure 2). However within a week after transplantation the patient had a life threatening hemorrhagic shock, in which FGF-21 increased severely. At her second and third admission with epilepsy a relatively mild rise in FGF-21 was seen.

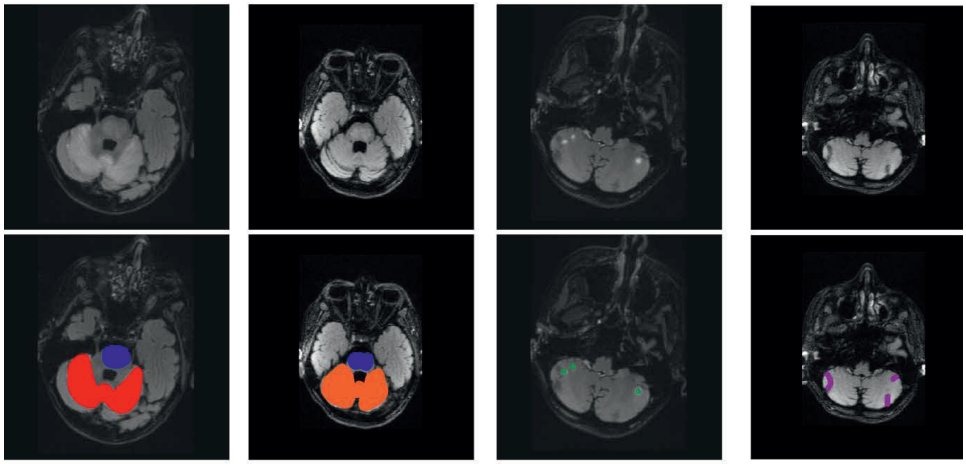
FIGURE 1. Brain MRI images of the two patients from our center. POD 83= initial MRI or CT-scan and POD 193 = follow-up MRI. T2= T2 weighted images. FLAIR = Fluid attenuated inversion recovery images, acquired after intravenous administration of contrast medium. T1 gado = T1-weighted image after gadolinium administration. 1a = case 1 and 1b = case 2.



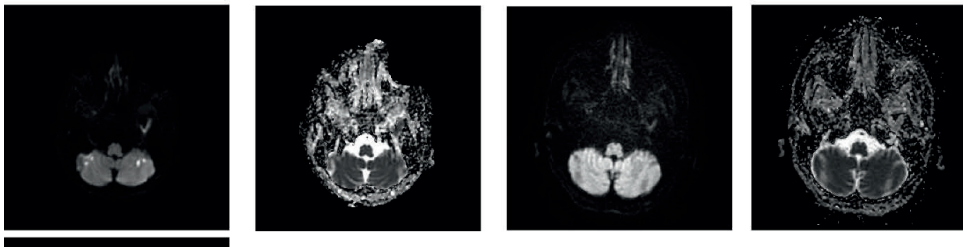
1a1 Initial MRI: swelling and T2 hyperintensity of basal ganglia (blue); lentiform fork (red); faint contrast enhancement (green); high intensity on DWI and low/normal ADC in basal ganglia (yellow). Follow-up MRI: decreased signal abnormalities of basal ganglia, with tissue loss.



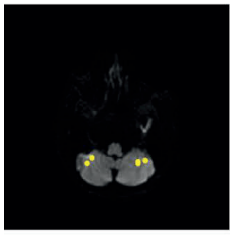
1a2 Initial MRI: swelling and T2 hyperintensity of mammillary bodies (blue); avid contrast enhancement (red) and no diffusion restriction. Follow-up MRI: decreased signal abnormalities, tissue loss.



MRI POD 83, FLAIR | MRI POD 193, FLAIR | MRI POD 83, FLAIR | MRI POD 193, FLAIR

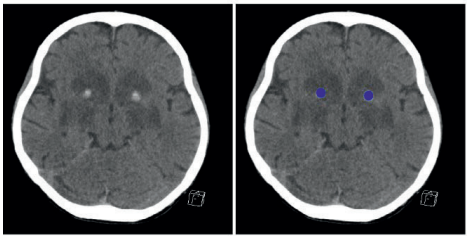


MRI POD 83, ADC | MRI POD 193, DWI | MRI POD 193, ADC



MRI POD 83, DWI

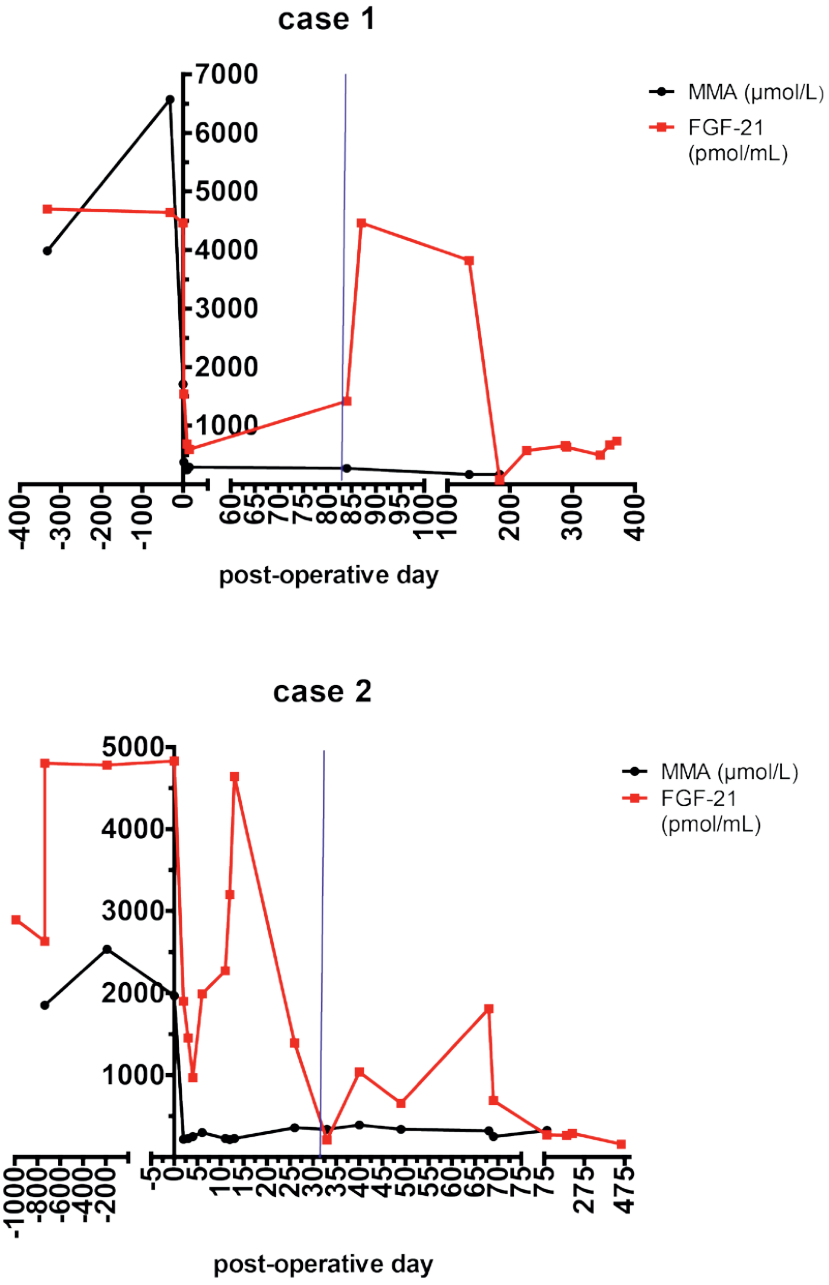
1a3 Pons: central T2 hyperintensity (blue). Upper cerebellum, SCA territory: diffuse T2 hyperintensity and swelling (red). PICA and AICA territory: asymmetrical focal T2 hyperintensities (green). Pons and SCA territory: no diffusion restriction (not shown). Focal lesions in PICA and AICA territory: diffusion restriction (yellow). Follow-up MRI: Pons: central T2 hyperintensity (blue); SCA territory decreased signal abnormalities (orange), diffuse atrophy; PICA and AICA territory: focal tissue loss (purple).



CT POD 83

1a4 Calcifications in anterior limb of internal capsule (blue).

FIGURE 2. FGF-21 and mma plasma level before and after transplantation in case 1 (a) and case 2 (b).



Blue vertical lines indicate timing of first neurological symptoms due to the CNI.

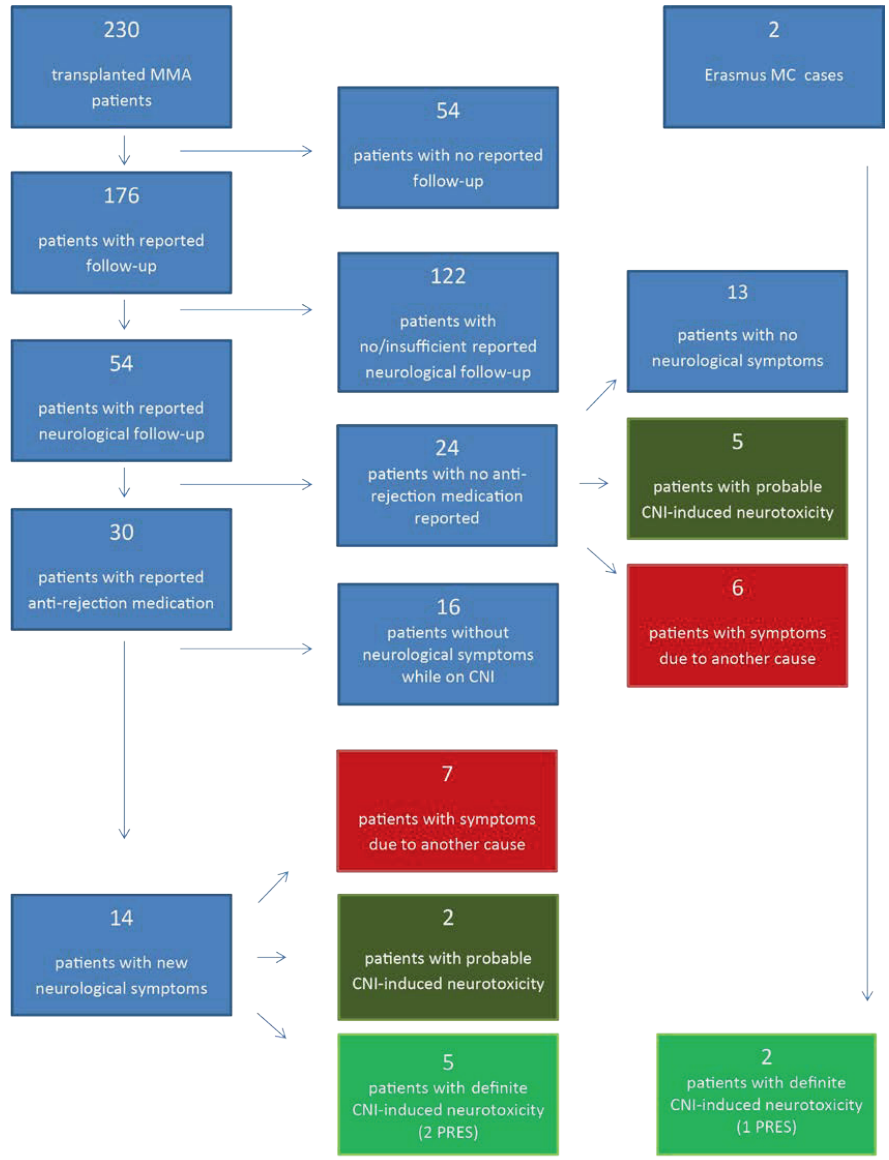
3.2 | Literature review

A total of 399 unique articles were retrieved and screened on title and abstract. One hundred and twenty-four of these articles reported a total of 230 transplanted MMA patients. Only 23% of the cases (54/230) explicitly reported neurological follow-up. Of these 54 patients, 24 were excluded from further analysis because of lack of information on the use of post-transplant medication (figure 3). However of these 24 cases, who likely all used a CNI, 5 (21%) had probable CNI-induced neurotoxicity (supplementary table 2a, dark green boxes figure 3) and 6 (25%) had acquired new neurological symptoms due to another cause (supplementary table 3a); the remaining patients did not develop new neurological symptoms after transplantation.

Of the 30 cases that were included in our analysis all were reported to receive a CNI. Sixteen patients (55%) did not develop new neurological symptoms while on CNI, five patients (17%) had definite CNI neurotoxicity (table 1, bright green boxed figure 3, (1 patient related to cyclosporine [23], and all others to tacrolimus), two patients (7%) had probable CNI-induced neurotoxicity and seven patients (23%) developed new neurological symptoms due to another cause (supplementary table 2b, 3b). Of the two patients with probable CNI-induced neurotoxicity (dark green boxes figure 3), one patients did not improve when tacrolimus was switched to cyclosporine (another CNI) and in the other patient no information regarding continuation of CNI was available.

Of the five patients with definite CNI neurotoxicity described in literature three had brain MRI-scan findings reported [12, 23, 24]. Signs of vasogenic edema were found in 2 patients (supplementary table 1); one of these patients had posterior cerebral involvement. Both patients were defined as having PRES considering the improvement of MRI findings and return of symptoms to baseline neurological status after discontinuing CNI respectively [23, 24].

FIGURE 3. Flow-chart of literature review and overview of excluded patients (which are further described supplementary table 1, 2a and b and 3a and b)



3.3 | Pooled incidence of CNI-induced neurotoxicity, including PRES

In total, 5 of 30 patients from the literature, and our two cases have been described in enough detail to conclude that they had CNI-induced neuro-toxicity (figure 3, table 1) resulting in pooled incidence of 22% (7/32) in MMA patients after solid organ transplantation, and an incidence of neurological symptoms due to other etiology than CNI of 22% (7/32) (supplementary table 3b).

Overall, in patients with CNI-induced neurotoxicity median age at transplantation was 20 years (range: 5- 28years) and the median follow-up after transplantation was 1.5 years (range 0.33-11.5year). Symptoms occurred between POD10 to 3 months after transplantation (table 1). Symptoms varied widely (table 1), with seizures being the most frequently presenting symptom (5/7). The majority of patients had tacrolimus levels within reported therapeutic reference ranges (5/6). Overall, 9% (3/32) of the patients likely had a CNI-induced PRES identified by brain MRI-scan.

4 | Discussion

This is the first systematic review that investigates the frequency of new neurological symptoms in MMA patients after liver and/or kidney transplantation and investigates whether these symptoms could be related to CNI-induced neurotoxicity, including PRES. Since neurological symptoms are likely to resolve when CNI is timely reduced/discontinued it is essential that physicians treating transplanted MMA patients are aware of CNI-induced neurotoxicity.

4.1 | CNI-induced neurotoxicity, including PRES, in transplanted MMA patients

4.1.1 | Clinical findings

According to our findings the risk of developing CNI-induced neurotoxicity in transplanted MMA patients seems similar to the risk of neurological symptoms due to other etiologies (22%), such a metabolic stroke-like episode. Differentiation between these different etiologies is difficult. The time after transplantation of occurrence of new neurological symptoms seems

to be indicative. CNI-induced neurological symptoms generally occur shortly after transplantation (days up to months [19, 25]. This is confirmed in this study (all within 3 month after transplantation), while symptoms occurring more than a year after transplantation seem to be more likely metabolically induced, among other probably due to the entrapment of mma in the brain [26]. To gain more insight in the effect of transplantation on mma levels in CSF and to aid clinical interpretation of new neurological symptoms after transplantation, we recommend the measurement of mma CSF levels before/during and after transplantation in all to be transplanted MMA patients.

However, in non-MMA patients CNI induced PRES has also been described years after kidney transplantation [27]; and CNI induced neurotoxicity should therefore never be discarded as possible cause. CNI induced neurotoxicity frequently presents with seizures, both in transplanted MMA patients (5/7 with CNI-induced neurotoxicity and 3/3 with PRES and in the majority of non-MMA patients with CNI-induced PRES [17, 19, 28]. We recommend considering CNI-induced neurotoxicity in all transplanted MMA patients presenting with new onset of seizures after transplantation. In CNI-induced seizures more recently developed anticonvulsants should be used and no valproic acid [17].

4.1.2 | Diagnosis and treatment

There are three steps to diagnose new neurological symptoms as CNI-induced neurotoxicity (in which plasma CNI levels do not necessarily have to be above the therapeutic window [25, 29, 30], including PRES [17]. Firstly, other etiologies should be excluded, such as a metabolic stroke-like episode, which is likely to be preceded by a systemic decompensation. Secondly, neuro-imaging should be performed. In case T2 hyperintensity is found on MRI, diffusion-weighted imaging (DWI) can help distinguish between vasogenic edema, which is typically found in PRES, and cytotoxic edema (with restricted diffusion) that is typical for acute ischemic stroke. However this is not always the case: restricted diffusion can also be encountered in PRES, and vasogenic edema can also occur in acute metabolic ischemic stroke. Brain location seemed typical only in one patient involving occipitoparietal region (Giussani). A recent study describes a central variant of PRES [31], which seems more in line with case 1. Thirdly, there should be improvement of symptoms after reduction /discontinuation of the CNI. There are

no clear recommendations on whether to reduce the dose or discontinue the CNI and how long discontinuation should last [21].

4.1.3 | Outcome

With early recognition and treatment of CNI-induced neurotoxicity, including PRES, the majority of patients will have no remaining neurological symptoms [28]. However, life-threatening complications may occur [32] and acquired neurological damage may persist [33, 34]. In the three MMA patients with PRES some remaining clinical abnormalities were found (case 1; [12, 24].

4.2 | FGF-21 and CNI-induced neurotoxicity, including PRES, in transplanted MMA patients

The exact pathophysiology of CNI-induced neurotoxicity, including PRES, is unclear but seems multifactorial [35] [36]. Impairment of oxidative phosphorylation by CNIs and thereby mitochondrial dysfunction has been reported to be a possible cause [37, 38]. Mitochondrial dysfunction plays an important role in the pathophysiology of MMA, and FGF-21 is a biomarker for mitochondrial dysfunction. Clearly increased FGF-21 levels have been described in MMA [22, 39-42]. We confirmed the severe initial decrease of FGF-21 after transplantation as recently reported by Manoli et al [43] in our two case reports. However, we also showed an increase in FGF-21 plasma levels in case 1 at the time of neurological symptoms due to CNI-induced PRES with a decrease in FGF-21 with improvement of symptoms and brain MRI-scan findings. This is suggestive for the pathogenic role of disturbed mitochondrial function in CNI-induced PRES. In case 2, a severe increase of FGF-21 was seen during hemorrhagic shock, which indicates that other factors such as ischemia impair mitochondrial function as well. Importantly, further studies are required on the potential role of FGF-21 in adding evidence to the underlying cause of CNI-induced neurotoxicity in transplanted MMA patients with new neurological symptoms. We hypothesize that patients with pre-existent mitochondrial dysfunction (as present in patients with MMA) are more at risk to develop CNI-induced neurotoxicity than the general population because of the cumulative effect of CNI on the

mitochondria. However, to date there is no clear evidence to support this and this should be prospectively investigated. If MMA patients indeed have a greater risk to develop CNI induced neurotoxicity than the general population we should consider to give a none CNI immunosuppressant, like Sirolimus, as first line immunosuppression in these patients, even though they are less potent as immunosuppressant [44, 45].

4.3 | Incidence of CNI-induced neurotoxicity, including PRES, in non-MMA transplanted patients

Previous studies in non-MMA transplanted patients reported an incidence of CNI induced neurotoxicity in 20% of the transplanted adults [46] and 8% of pediatric patients [47, 48]. The incidence of CNI induced PRES has been reported to range from 0.34 to 1% of the non-MMA patients after transplantation [19] [25] [27]. The incidence of CNI-induced neurotoxicity in transplanted MMA patients in this study seems comparable to the incidence with non-MMA transplanted patients, while the incidence of PRES seems much higher than in non-MMA transplanted patients.

4.4 | Study limitations

Several limitations of this study require attention. The true incidence of CNI-induced neurotoxicity is difficult to investigate by means of a systemic review since not all transplanted MMA patients have been published, neurological follow-up was described only in a minority (20%) of the reported cases and it is uncertain whether the authors/clinicians were aware of the possibility of CNI-induced neurotoxicity, including PRES, in MMA. Adding our patients to the total of transplanted patients could lead to bias. However since these cases were the only two MMA patients transplanted within our center we avoided this bias as much as possible. Furthermore, several authors published numerous articles with the likelihood of using the same patient case multiple times without explicitly mentioning so. We did therefore look into potential duplicates.

In order to investigate the true incidence of CNI-induced neurotoxicity, including PRES, and neurological complications due to other causes it is essential to establish a thorough prospective database with follow-up of all the MMA transplanted patients. Since pre-existent MRI abnormalities are frequent in MMA patients a pre-transplant brain MRI-scan would be of great value to distinguish between pre-existing and new problems in all (to be) transplanted MMA patients. In MMA patients typical MRI findings (with typical as well as atypical brain locations) suggest PRES with the added value of DWI and ADC (in first 10 days after new neurological symptoms). Furthermore, mma levels and FGF-21 in plasma and cerebrospinal fluid should be included in future studies/databases.

5 | Conclusion

CNI-induced neurotoxicity should be considered in all transplanted MMA patients with CNI use and new neurological symptoms. When CNI-induced neurotoxicity cannot be excluded brain MRI-scans (including DWI) should be performed and CNI dose must be reduced or discontinued, since with timely recognition of CNI-induced neurotoxicity and dose reduction or discontinuing CNI, symptoms can resolve.

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Supplemental tables and figures

Supplementary table 1. MRI abnormalities in those with CNI-induced PRES.

Case	Case 1 (see also figure 1)	Vernon	Giusanni
CNI start, **CNI stop,** **CNI restart**	POD1, POD 86, No	POD6, POD28, No	POD1, POD10, POD12 at lower dose
MRI/ CT-scan findings:			
Basal ganglia	Post Tx MRI: POD 83: Symmetrical swelling, T2 hyperintensity, lentiform fork sign, high signal intensity on DWI with intermediate ADC, mild contrast enhancement Follow-up Tx MRI: POD 193: Decreased signal abnormalities, no more contrast enhancement, tissue loss	Post Tx MRI including MRA: POD 4: Small T2-hyperintese lesions in the posterior aspect of the globi pallidi which were felt to be a sequelae of her prior infarct, unchanged on Follow-up Tx POD 28	
Mammillary bodies	Post Tx MRI: POD 83: Symmetrical severe swelling and T2 hyperintensity, avid contrast enhancement, without diffusion restriction Follow-up Tx MRI: POD 193: Decreased signal abnormalities, no more contrast enhancement, tissue loss		
Truncus cerebri	Post Tx MRI: POD 83: T2 hyperintensity pons without diffusion restriction or contrast enhancement, probably due to postischemic changes Follow-up Tx: POD 193: conform	Follow-up Tx MRI: POD 28: Mesencephalon and pons: Patchy T2 hyperintense signal in the pons and left midbrain with matching increased apparent diffusion coefficient (ADC) values Follow-up Tx MRI: POD 48: Improvement in the previously visualized changes in the pons on T2 weighted images.	
Cerebellum	Post Tx MRI: POD 83: Symmetrical swelling, T2 hyperintensity, SCA territory without diffusion restriction. Asymmetrical focal T2		

	hyperintensities with diffusion restriction PICA and AICA territory		
Case	*Case 1 (see also figure 1)*	*Vernon*	*Giusanni*
Cerebellum	Follow-up Tx MRI: POD 193: Decreased signal abnormalities, atrophySCA area Focal tissue losses PICA and AICA area without signal abnormalities of the remaining parenchyma		
Cingulate gyrus		Follow-up MRI: POD 48: There was minimally increased abnormal signal in the right cingulate gyrus with reduced diffusion on ADC maps	
Diencephalon		Follow-up Tx MRI: POD 28: Small T2 hyperintense lesions in the left thalamus without matching diffusion abnormalities Follow-up Tx MRI: POD 48: Improvement in the previously visualized changes in the thalamus on T2 weighted images	
White matter		Follow-up Tx MRI: POD 28: Small T2 hyperintense lesions left corona radiate without matching diffusion abnormalities	Post Tx CT: POD 10: Subcortical hypodensities in the left temporal, right parietal, and bilateral occipital regions Post Tx MRI: POD 10: Multiple large areas of vasogenic edema in the occipitoparietal and frontotemporal regions on T2 and FLAIR images with no diffusion restriction
Capsula interna	Post Tx MRI and CT-scan: POD 83: Anterior part calcifications Follow-up Tx MRI: POD 193: unchanged		
Other:		Post Tx MRI: POD 4: T2 hyperintense signal of the orbital segment of both optic nerves, and mild reduction in size of the	

			right side of the optic chiasm, consistent with acute optic nerve damage superimposed on a chronic bilateral optic neuropathy	

Abbreviations: Tx= transplantation, POD= postoperative day.

Supplementary table 2a. Patients with reported neurologic follow-up but without reported medication (n=5), which were probable CNI-induced neurotoxicity.

Case (n=)	Follow-up duration	Age at transplantation	Time after transplantation and symptoms	MRI
Hoff (n=1)	*4y6mo*	*13y6mo*	*<3month* *Seizures and intracranial hemorrhage*	*un*
Kasahara (n=3)	*un*	*un*	*un* *New onset of seizures (3x)*	*un*
Yoshino (n=1)	*2y7mo*	*7y3m0*	*POD 19* *Episodes of quick torsional movements of head* *(2y7mo* *Developed an episode of tonic seizures)*	*un*

Abbreviations: n= number of patients; y=year; mo=month; un= unavailable; POD=postoperative day.

Supplementary table 2b. Patients with reported neurologic follow-up while on CNI (n=2), which were probable CNI-induced neurotoxicity.

Case (n=)	Follow-up duration	Age at transplantation (type of transplantation)	Time after transplantation and symptoms	MRI	Tacrolimus plasma levels	Outcome
Kaplan (n=1)	*8y5mo*	*19mo (LT)*	*POD30* *Severe, coarse, generalized tremors*	*POD 72* *MRI: an acute lesion in the right globus pallidus, consistent with ischemic and/or edematous changes* *Subsequent MRI 18 months later showed resolution of the basal ganglion lesion.*	*Within therapeutic range*	*Tacrolimus discontinued and cyclosporine started; no clear improvement of tremors (no information on time period given); subsequent reinstitution of tacrolimus and discontinuation of cyclosporine produced no immediate/apparent worsening (no information on time period given); treated with oral clonazepam therapy with gradual improvement. The tremors resolved after 1 year*
Burlina (n=1)	*un*	*18y (KT)*	*4mo* *Partial motor seizures with secondary generalization, without biochemical decompensation*	*DW-MRI: Hyperintense bilateral lesions tegmentum of the pons.* *MRI: confirmed the stroke-like lesions in the same areas*	*un*	*Antiepileptic therapy was started, no further outcome reported, no information on whether CNI was lowered or discontinued*

Abbreviations: n= number of patients; y= year; mo=month; un=unavailable; POD=postoperative day

Supplementary table 3a. Patients with neurotoxicity due to another cause without reported medication

Case (n=)	Current age of patient	Age at transplantation (type of transplantation)	Time after transplantation and symptoms	CNI applied	MRI
Chakrapani (n=1)	*5y, 9mo*	*9mo* *(LT)*	*NA (after surgery)* *Seizures while having metabolic acidosis and hyperammonemia* *5y* *Had an unexpected episode with sudden onset of altered consciousness, loss of speech, and hypotonia.*	*un*	*un* *CT unremarkable. Repeat neuroimaging 1 week after the acute episode revealed bilateral basal ganglia changes*
Khan (n=1)	*31y*	*Early in life* *(2x LKT)*	*NA (at 20y of age)* *Haemorrhaic stroke*	*un*	*un*
Shenoy (n=1)	*un*	*NA* *(KT)*	*2 mo* *Bacterial endocarditis and then progressive deterioration in graft function.* *(13 mo* *Episode of pancreatitis and neurological deterioration care was withdrawn)*	*un*	*un*
Nakajima (n=1)	*5y4mo*	*1y7mo* *(LT)*	*1y7mo* *Disability, altered consciousness, fever of unknown origin*	*un*	*MRI, MRS indicated Leigh's encephalopathy*
Yoshino (n=1)	*5y2mo*	*2y* *(LT)*	*2y* *Weakness of right extremities and flexion of right upper extremity*	*un*	*un*
Sissaoui (n=1)	*un*	*un* *(LKT)*	*NA* *Axonal neuropathy and myoclonus*	*un*	*un*

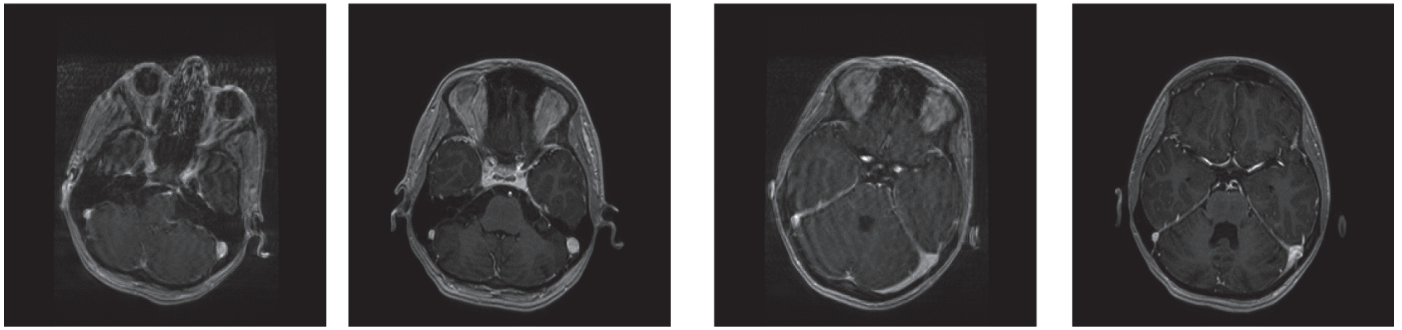
Abbreviations: n=number of patients; LT = liver transplant; KT= kidney transplant; LKT= liver and kidney transplant; CNI= calcineurin inhibitor; y = reported time after transplant in years; mo=months; POD = postoperative day; un = unavailable.

Supplementary table 3b. Patients with likely non-CNI induced neurotoxicity with reported medication (CNI)

Case (n=1)	Current age of patient	Age at transplantation (organ)	Time after transplantation and symptoms	CNI applied	MRI
Clothier (n=1)	*16y*	*12y (KT)*	*un* *Generalized sensory motor peripheral neuropathy,*	*yes*	*MRI scans of his brain at ages 14 and 16 revealed long-standing bilateral changes in the globus pallid and no other significant abnormalities*
Khanna (n=1)	*28y*	*24y (LT)*	*POD 183-191* *Had a seizure episode and purulent meningitis*	*yes*	*un*
Brassier (n=1)	*8y*	*11y*	*18mo* *Neurological regression with hepatoblastoma*	*yes*	*Brain MRI showed bilateral pallidal lesions, hypersignal of dentae nuclei, vermian atrophy and cerebral edema*
Kasahara (n=1)	*un*	*9mo (LT)*	*5y* *Severe neurological insult while recovering from chest infection without any systemic disturbance*	*yes*	*un*
Kasahara (n=2)	*un*	*22y and 13y respectively (both LT)*	*un* *Both progressive neurological disability*	*yes (2x)*	*un*
Nyhan (n=1)	*un*	*22y*	*un* *Progressive neurologic abnormality; developed acute spasmodic contractions*	*yes*	*CT and MRI of the brain were normal; there were no lesions in the basal ganglia or white matter.*

Abbreviations: n=number of patients; LT = liver transplant; KT= kidney transplant; LKT= liver and kidney transplant; CNI= calcineurin inhibitor; y= reported time after transplant in years; mo=months; POD, postoperative day; un = unavailable.

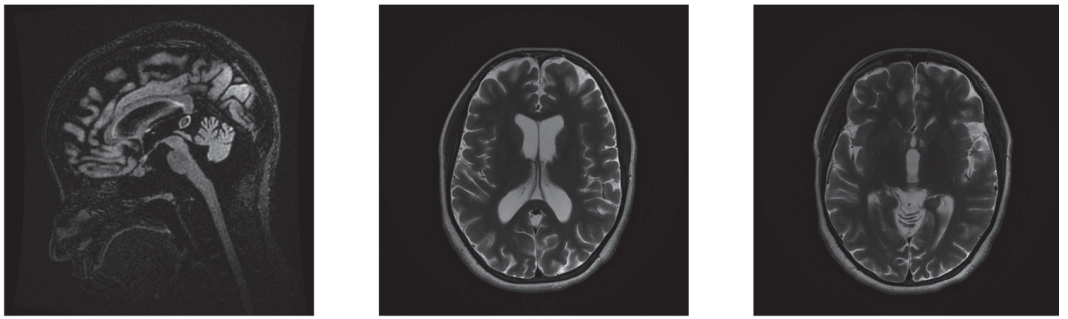
Supplementary figure 1. Brain MRI images of case 1 from our center.



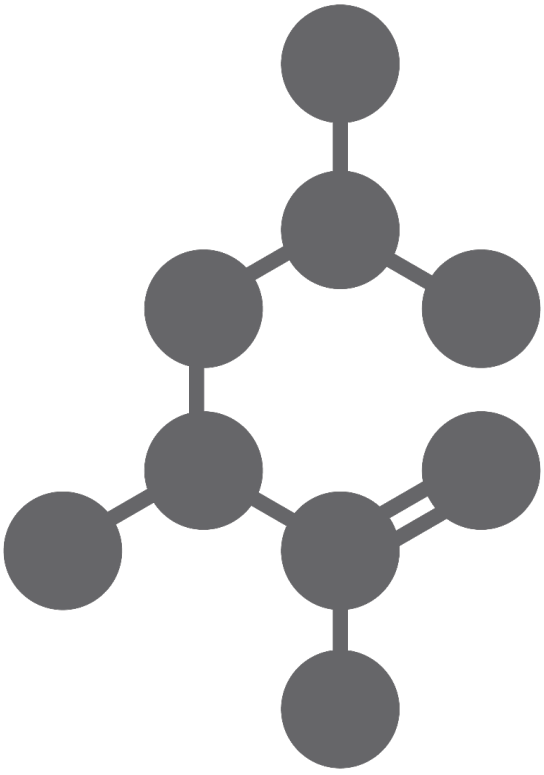
MRI POD 83, post contrast | MRI POD 193, post contrast | MRI POD 83, post contrast | MRI POD 193, post contrast

POD 83= initial MRI or CT-scan and POD 193 = follow-up MRI. Post-contrast images shown.

Supplementary figure 2. Brain MRI images of case 2 from our center.



Vermis hypoplasia, enlarged fourth ventricle.



GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In recent years treatment for classical organic acidemia (OAD) and urea-cycle disorders (UCD) has not changed dramatically regarding the dietary therapies. It is known that with the currently applied treatment, patients have an unsatisfactory outcome characterized by recurrent metabolic decompensations, long-term complications (with an increased mortality risk), impaired growth, and cognitive developmental delay. The data collected by the E-IMD (n=271 OAD and n=361 UCD patients) and the collection of a large retrospective cohort in the Netherlands on methylmalonic (MMA) and propionic acidemia (PA) patients (n=76 patients) made it possible to analyze current dietary treatment strategies and define how to improve these. Furthermore, the effort of all European metabolic clinicians led to the collection of a large data set on liver and/or kidney transplantation performed in Europe.

The hypothesis to be answered was: patient's outcome can be improved by optimization of the currently available treatment. It is essential to evaluate currently applied treatment strategies, not only because long-term outcome of classical OAD and UCD patients continues to be disappointing, but also because newborn screening for these diseases is implemented in an increasing number of countries.

This thesis had four sub-aims:

1. To explore the natural history of classical OAD and UCD patients and the treatments applied, to provide insight in the outcome/prognosis obtained with the currently applied therapies (chapter **2, 3** and **6**).
2. To determine the most optimal dietary treatment advice (chapter **2** and **4**).
3. To improve monitoring of classical OAD and UCD patients (chapter **5** and **6**).
4. To determine the outcome (mortality, complications, quality of life (QOL) and cognitive development) of AOA patients who received solid organ transplantations (chapter **7**).

This chapter discusses the main findings of the research performed and described in this thesis and their relevance to clinical practice. Firstly, the outcome measures will be argued, and thereafter treatment recommendations and tools on how to monitor patients will be provided.

MAIN CONCLUSIONS OF THIS THESIS

Discussed in 1.1 and 1.2:

1. Despite their relatively high natural protein prescription according to WHO recommendations, classical OAD and UCD patients have low levels and disturbed ratios of branched chain amino acids (BCAA) (chapter 2 and 4).
2. In classical OAD patients, and in particular in MMA, amino acid mixtures can be harmful and should be prescribed as advised and with care (chapter 2 and 4).

Discussed in 1.3:

1. Impaired growth is observed; optimization of L-arginine in MMA and PA and of branched chain amino acid levels in UCD is essential to improve growth (chapter 3). Height is associated with the natural and total protein energy ratio (chapter 3 and 4).

Discussed in 1.4:

1. Newborn screening in MMA and PA can potentially lead to a reduction of an adverse neurological outcome from 46% to 36%. The frequency of decompensations, mitochondrial complications and treatment related complications are most likely not positively affected (chapter 6).

Discussed in 2.0:

1. Metabolic decompensations and long-term (mitochondrial) complications are major problems in MMA and PA patients. The high protein prescription increases the risk of metabolic decompensations (PA) and mitochondrial complications (MMA) and of impaired cognitive development (entire MMA and PA cohort) (chapter 4).
2. In MMA and PA patients, long-term mitochondrial complications can be life threatening; FGF-21 is a good biomarker to define risk groups for mitochondrial long-term mitochondrial complications (chapter 5).
3. Liver and/or kidney transplantation in AOA seems a safe treatment option with good overall survival (78-100%) and stable or improved cognition and quality of life (chapter 7.1). However in MMA patients, caution should be taken in using calcineurin inhibitors (CNI), since they can induce severe neurotoxicity, likely due to pre-existing mitochondrial dysfunction (shown by FGF-21 levels). This neurotoxicity can be reversible when CNI is stopped in time (chapter 7.2).

Discussed in 3.0:

1. To improve patient care of MMA and PA patients identified by the recently implemented newborn screening program, additional recommendations on clinical evaluation for long-term complications are given (chapter 4 and 6).

1 | Main outcomes regarding dietary treatment

1.1 | A high protein intake is prescribed and (in MMA and PA) harmful

Protein prescription in classical OAD

The guidelines for MMA and PA patients states that: '' The goals of long-term management are to achieve normal development and to prevent episodes of metabolic decompensation, whilst providing good quality of life and avoiding side effects and complications''. In the European cohort, we observed that several patients had a high natural protein prescription and total protein prescription, even above RDA by the FAO/WHO/UNU. In OAD, the guideline suggests prescribing AAM-OAD in those patients with a natural protein prescription below the RDA. However, we observed that the majority of those who received AAM-OAD, had natural protein prescription that was already compliant and even above the recommended daily allowances (RDA). In all subgroups of MMA and PA patients, total protein prescription was high according to the RDA, even in the severe patients (MMA vitaminB12 unresponsive and PA early onset (EO) patients). In the severe patients, the high total protein prescription was mainly due to the prescription of AAM-OAD. Why such high amounts of protein are applied remains unclear. It could be due to 1) unfamiliarity with RDA, as most often g/kg is used to calculate protein prescription, 2) adjustments of protein prescription when impaired growth is observed, 3) prescribing more than RDA to compensate for the protein quality patients consume, or 4) adjustments of protein prescription based on low BCAA levels.

Whether or not the natural protein prescription could be further increased was unknown and a trend towards prescribing a higher natural protein in the meantime was already taking place. In the sub-cohort of the Dutch MMA and PA patients (chapter 4) we clearly showed that an increased natural protein prescription increases the risk of 1) metabolic decompensations, 2) mitochondrial complications, and 3) impaired cognitive development. This holds in particular for the severe disease types (MMA vitaminB12 unresponsive and PA EO patients). Firstly, based on the pathophysiology a higher tendency towards metabolic decompensations seems likely with higher protein prescription. However, to date it was unclear at which protein prescription this risk on decompensation was significantly increased. It is necessary to take patient specific characteristics into account, such as physical activity. Secondly, more metabolites that are toxic seem to accumulate with high protein prescription resulting in a higher detrimental effect on

mitochondrial function. These metabolites can harm the mitochondria and thereby disturb their function. The harmful effect on mitochondrial function will be further explained in the latter part of this discussion. Thirdly, regarding cognitive development it is known that certain proteins can affect the integrity of the blood brain barrier and thereby induce stress on neurological functions [1].

Regarding protein prescription in MMA and PA patients the statement: 'first do no harm', seems applicable. Which mean that to date it is recommend to prescribe a lower protein intake, to reduce metabolic decompensations, mitochondrial complications and potentially improve cognitive development. It should be taken into consideration that one should compensate for a low biological value of the protein consumed. For MMA vitB12 responsive patients, it is recommended not to prescribe AAM to any of these patients. In MMA vitB12 unresponsive patients, outcome needs to be evaluated in patients treated with a natural and total protein prescription below RDA. It needs to be answered what will happen with the BCAA plasma levels and with growth. This prospective evaluation will take a long time-period and therefore other studies are essential. To obtain an answer on the short-term in vitro studies might help to get insight in the effect of adjusted dietary therapies on cellular levels and in the potential pathophysiological mechanisms that elicit the harmful effects. Furthermore, we advise to measure biomarkers (FGF-21 and potentially GDF-15) when dietary therapies are adjusted in patients, to monitor the potential harmful effect on mitochondria in patients. Potentially, it should be advices that protein in MMA and PA patients need to be of low as well as high biological value, as was recommended recently for UCD patients in the newly published UCD guideline [2].

Protein prescription in UCD

The goal of the dietary treatment in UCD is to acquire metabolic stability with optimized growth and development, while preventing chronic complications [2]. The guideline for UCD proposed by Häberle et al [2, 3] suggests that the FAO/WHO/UNU requirements should be used to determine optimal protein prescription. The protein RDA by the FAO/WHO/UNU is based on individuals consuming protein of a high biological value. The protein commonly used by UCD is however often of low biological value [4]. The guideline recommends, when

synthetic protein (AAM-UCD) is used, to prescribe 20-30% of the total protein prescription as protein from AAM-UCD [3]. The updated guideline also discusses that the effects of physical activity of patients on their protein needs, need to be taken into account [2]. In this thesis it is shown that natural protein prescription in UCD patients was compliant with the RDA in several patients, but that total protein prescription was above RDA in the majority of patients due to the additional amino acid supplementation. There was no clear difference in total protein prescription between the UCD subgroups. The differences between countries regarding protein prescription, clearly indicates that to date it is unknown what optimal dietary prescription in these patients is.

AAM-UCD should be applied in patients with a low protein tolerance. Accordingly, in this thesis it is shown that in numerous symptomatic patients taking additional AAM-UCD, natural protein prescription was below the RDA, while total protein prescription was compliant to and above the RDA. In UCD, AAM-UCD contain essential amino acids and these products seem efficient in acquiring higher plasma amino acid levels. The products should not include tryptophan, phenylalanine, and tyrosine. These amino acids can be used to produce serotonin and dopamine, which levels can be increased in hyperammonemia and they can be therefore harmful for the patients. So far, AAM-UCD products have not been shown to cause any harmful effects and the advice is to prescribe these products in patients with low plasma BCAA levels, low protein prescription and/or sodium phenylbutyrate use.

The amount of protein prescribed in UCD seems sufficient according to guidelines, but it is questionable if this advice is indeed appropriate and whether optimal patient outcome is achieved with this treatment. Overall protein prescription compared to the RDA was relatively high. And a high protein prescription can be harmful since it can induce hyperammonemia and put the patient at risk for renal disease [5]. It is interesting to observe that mainly asymptomatic patients received a high total protein prescription and it is unknown why this is done. It could well be that clinicians are fearful to induce deficiencies by a relatively low protein diet. A low protein intake can induce mineral and vitamin deficiencies and physicians should monitor this and when necessary supply minerals and vitamins to patients. However, to date there is no evidence to support that a lower protein presription in these patients will be actually harmful. The protein that is consumed should, according to the last insights, contain protein of high as well as low biological value to avoid any deficiencies.

More in depth studies, such as done in the sub-cohort of Dutch MMA and PA patients, are necessary to evaluate the potential harmful consequences of the high total protein prescription. To date, we recommend close monitoring of classical OAD and UCD patients, of their outcome and of their plasma amino acid levels. A biomarker to monitor disease progression is essential to optimize patient outcome.

1.2 | Low plasma BCAA levels are frequently observed

BCAA (and aromatic neutral amino acids) compete for the cellular uptake by the same L-type amino acid transporter (LAT) [6]. Four subtypes of LAT are known, all of them are involved in the transport of BCAA and methionine and phenylalanine. LAT1 and LAT2 are also transporting other amino acids such as histidine and tyrosine. LAT1 is located at the blood-brain barrier, colon, and liver. Interestingly, LAT1 is also expressed at the blood retinal barrier and we should look into this transporter (including the BCAA transport) in our patients/mouse models regarding the occurrence of optical atrophy in classical OAD patients. LAT2 is expressed in the kidney, jejunum, ileum, but also on brain and liver. Not only amino acids are transported by the LAT transporter, but thyroid hormones as well (by the LAT1 and LAT2). Interestingly, in some MMA and PA patients disturbed thyroid hormone levels have been observed with to date no clear pathophysiological explanation. LAT3 is expressed in human pancreas, liver, skeletal muscle, and fetal liver, whereas LAT4 is expressed in placenta, kidney, and peripheral blood leukocytes.

Disturbed BCAA ratios can influence the uptake of any of the BCAA by the LAT. High levels of leucine can lead to a decreased uptake of the other two branched chain amino acids and isoleucine as well as valine can both interfere with leucine uptake. Optimized BCAA ratios will most likely improve BCAA uptake.

In MMA and PA, plasma L-valine and L-isoleucine levels lay below reference ranges in respectively 57% and 55% of the patients in the E-IMD cohort. We confirmed these low BCAA levels in the Dutch sub-cohort of MMA and PA patients. In MMA and PA patients, AAM-OAD products seem to partially cause the disturbed BCAA ratios. AAM-OAD contain high amounts of leucine, while no valine or isoleucine. In MMA and PA we showed that L-leucine: L-isoleucine

as well as L-leucine: L-valine ratios were higher in those who received AAM-OAD versus those who did not, which is in line with a study by Myles et al. [7].

BCAAs are essential to 1) maintain anabolism [8], 2) optimize growth (height) and bone marrow function [9, 10], 3) support normal development [11-13], 4) optimize functioning of the central nervous system [14-16], and 5) support normal mitochondrial function [17]. Towards improving patient care, it thus seems necessary to fine-tune plasma BCAA levels in individual patients. The first step towards better monitoring of the classical OAD patient is regular measuring of plasma BCAA levels [18].

In UCD, plasma L-valine, L-isoleucine and L-leucine levels lay below reference ranges in 18%, 30% and 31% of the total group of UCD patients in the E-IMD cohort. The cause for the low plasma BCAA levels could be the protein restricted diet; however, we observed a relative high protein prescription in the majority of patients. AAM-UCD products contain BCAA in ratios that are closer to the optimal ratio of 1:2:4 (isoleucine: leucine: valine) than the products in AAM-OAD. Isoleucine content was a bit higher and valine contents overall a bit lower in AAM-UCD products. However, plasma BCAA ratio's observed in UCD patients consuming AAM were normal and AAM-UCD do not seem to be explain the low plasma BCAA levels in UCD patients. One important cause for low BCAA levels in UCD patients is the use of sodiumphenylbutyrate [8, 19] and patients using this medication should be monitored closely.

In MMA and PA, for optimized dietary treatment it is essential to calculate BCAA ratios, rather than to monitor their overall plasma levels only. In the first published study in this thesis on dietary prescription in classical OAD and UCD, we stated that since a low natural protein prescription can be potentially harmful, i.e. low BCAA levels, one should ensure that each patient achieves a higher natural protein prescription in such a way that it does not cause metabolic instability. Regarding AAM supplementation in MMA and PA, patients who received AAMs-OAD had very low BCAA levels and disturbed plasma BCAA ratios. Considering the likely harmful effects of AAM products on these BCAA plasma levels, it could be questioned what the effect of increasing natural protein prescription will be if a patient is still on AAM. In patients already receiving 100% RDA natural protein or in those with an adequate RDA we highly recommend to stop AAM prescription. In UCD patients, it seems that AAM can help to

obtain normal BCAA levels. In UCD, we recommend to apply AAM in those patients with low BCAA levels. Furthermore, in line with OAD, we must also pay attention to the BCAA ratios.

Pathological mechanisms in other diseases and the knowledge obtained from treatment in these diseases can potentially be applicable to the disorder focused upon in this thesis. For MMA and PA patients, we hereby examined the treatment of phenylketonuria (PKU) patients. PKU patients are treated with indispensable L-amino acids, which decrease plasma phenylalanine levels due to interaction on a the LAT1 carrier protein. Furthermore, it is known that this transporter present on the mucosal epithelium is the same as the one exposed in the brain and it is shown that phenylalanine levels in the brain are decreased by these L-amino acids [20-22]. In MMA and PA, the same LAT transporter plays a role on the mucosal epithelium and is also present at the blood brain barrier. In PKU, a clear recommendation is given on the amount of L-amino acid supplements that should be provided [23]. This principle of treating patients with a certain amino acid to counteract the uptake of the 'toxic' amino acids could be used in MMA and PA patients. Within the early years of discovery of MMA and PA, a study has been published on the effect of solely applying L-leucine to a patient [24]. The AAM-OAD products have a high level of leucine and considering the interaction it could be that it is helpful in decreasing isoleucine and valine uptake. However, according to our data it seems that with the current applied dietary treatment there is no appropriate balance in BCAA plasma levels, with very low levels of isoleucine and valine in the majority of the patients. In conclusion, it is definitely worth considering leucine supplementation as a treatment strategy, however in lower quantities than the high amount currently available in AAM products. Lower amounts might be helpful to obtain optimal BCAA ratios and uptake.

Regarding recommendations on plasma BCAA levels, which to date are not given in MMA and PA, the guidelines for MSUD could be of help. These guidelines give clear recommendations on plasma BCAA levels at certain ages (in case of leucine). In MSUD: ''The goal of dietary BCAA restriction for the individual with MSUD is to achieve and maintain plasma BCAA concentrations as close to normal as possible while preventing and correcting BCAA deficiencies''. While this has not mentioned to be a goal for MMA and PA patients, it could well be that we should achieve and maintain plasma BCAA levels close to normal considering

the very low BCAA levels in our patients. Hereby we aim to achieve normal BCAA ratios and potentially improve growth and decrease metabolic decompensations. Optimization of these BCAA levels rather than aiming towards a high total protein prescription could be a future goal of treatment. Furthermore this MSUD guideline states: 'to promote anabolism of LEU, when LEU blood concentrations are high, additional supplementation of VAL and ILE is often required'. So again, in both the PKU and the MSUD guideline it is recommended to take the interaction of BCAA with the same LAT transporter into account in the treatment of patients. In the MSUD guideline it is even stated how much of each BCAA has proven to be successful and should be taken as part of the daily nutrient prescription [25].

1.3 | Growth (height) is often impaired

Considering the dietary treatment in classical OAD and UCD patients, we have to be aware of the fine scale/balance the patients live on. Growth in children can be used as a 'marker' of good health. On one side we want to increase protein prescription to optimize growth, and on the other side we want to minimize the risk of long-term complications (such as mitochondrial complications) and metabolic decompensations. This all while preventing neurological damage and maintaining optimal cognitive development. Growth is disturbed in the majority of classical OAD and UCD patients [26-31]. While growth is often used as outcome measure for appropriate protein intake it is questionable whether this assumption is correct in these patients. It is furthermore questionable if normal height needs to be achieved in patients with these diseases. In our early studies, we suggested that optimal growth will result in improved protein tolerance and possibly a decrease in the number of decompensations. We must consider the possibility that in classical OAD and UCD patients not necessarily the total protein intake reflects growth, but that rather the deficiency of certain specific amino acids, such as L-leucine, L-isoleucine, L-valine, L-histidine, L-arginine and L-lysine are the cause of insufficient growth [11, 32]. Whether the disturbed growth is an important issue in these patients is questionable. Of course, with improved growth protein tolerance it will likely be optimized. If a higher protein prescription is necessary to achieve optimized growth, while inducing decompensation and mitochondrial complications optimized growth should not be the main goal of treatment.

In the study on growth in OAD patients, we observed that L-arginine plasma levels are associated with growth. While L-arginine supplementation can potentially be beneficial, it can also have severe side effects [33], such as the induction of hypoglycemia and excessive production of nitric oxide [34]. Further research should be performed with regard to recommendations on the most optimal plasma L-arginine levels in these patients and whether supplementation of L-arginine is a safe method. In UCD, disturbed growth was mainly observed in CPS1-D and ASS-D patients. Plasma L-leucine and L-valine levels in UCD patients were associated with growth in UCD subgroups and AAM-UCD can be helpful to improve growth.

Regarding protein prescription, the protein: energy ratio is often calculated and used in dietary prescription. In our studies we found a positive effect of increased P:E ratio on growth in MMA unresponsive patients in the subset of the Dutch MMA patients. Furthermore we observed that a higher P:E in PA EO patients was associated with less mitochondrial complications. A disturbed ratio can be due to a low protein prescription (although this was not observed in any of the published studies) or due to a high kilo caloric intake installed to avoid catabolism. While the P:E ratio seems helpful in dietary management it is essential to acknowledge that several factors need to be taken into account, which clarify that the use of P:E ratio has some drawbacks. Energy intake does not always reflect energy requirements. Energy requirement is highly dependent on physical activity. Therefore it is hard to define the optimal P:E needed to achieve optimal growth.

1.4 | Impaired cognitive development is regularly seen and associated with protein prescription (in MMA and PA)

Cognitive development is often impaired in classical OAD and UCD. Of the Dutch MMA and PA patient group almost one third had an IQ below 60. Cognitive development is more frequently impaired in PA patients than in MMA patients [35, 36]. The impaired cognitive development can be caused by different factors, of which 1) the timing and severity of the first metabolic decompensation, 2) recurrent decompensations and 3) ongoing toxicity are of main

importance. To avoid neurotoxicity induced by the first metabolic decompensation early recognition of the disease and immediate start of treatment is essential. In this thesis we showed that the introduction of newborn screening could potentially be beneficial in preventing neurological damage of first symptomatic presentation. However, studies performed so far show that newborn screening did not seem to influence the clinical course, including the number of metabolic crises, physical and neurocognitive development, and long-term complications [21,22]. This is in part due to the fact that there is currently no curative treatment available. It is essential to be aware that by implementing newborn screening we may lose the discrimination between patients with early and late onset of PA. By not knowing how the patient would have presented without NBS, it is harder to determine whether a patient will be severely affected or milder and thus which treatment will be appropriate. Identification of the genetic variants may help to discriminate. In case of an unknown variant functional in vitro studies (e.g. in vitro mutagenesis) may be helpful to predict the phenotype. One can speculate that biomarkers for disease severity become even more important now newborn screening has been implemented. In line with this further studies on FGF-21, in particular in patients identified by NBS, are essential to guide patient treatment and care.

Recurrent decompensations still occur despite current treatment strategies. In our study in Dutch MMA and PA patients, we observed that MMA vitaminB12 unresponsive and PA EO patients were mainly at risk of metabolic decompensations. Newborn screening in these patients did not seem beneficial on the severity/frequency of metabolic decompensations. Regarding treatment during and after decompensations several questions are still unanswered. For example, it is not clear whether patients need a higher protein prescription (than before admission to the hospital) after a metabolic decompensation to compensate for the temporarily lower intake (often already before and) during decompensations. It could well be that they do need some extra intake to recover from this decompensation [37]. The effect of decompensations on patient development, stability and energy balance needs to be taken into account.

Ongoing toxicity occurs due to accumulation of toxic metabolites even when a patient is stable and not decompensated. In PA, one of the toxic metabolites that accumulates and have shown to be associated with cognitive outcome is 3-hydroxypropionate [35]. Regarding the cognitive development, BCAA seem essential for optimal functioning of the central nervous system [11-

16]. As previously mentioned the LAT is the main transporter for BCAA, also for transport across the blood brain barrier. In this thesis we showed that a high protein prescription was associated with more severe cognitive developmental delay in MMA and PA patients. Different studies have been performed to determine the effect of proteins on brain function in other diseases. While it is known that BCAA are essential for cognitive development, it is also shown that a high protein intake can disturb the blood brain barrier function. Furthermore the oxidative stress/ mitochondrial dysfunction observed in MMA and PA patients can also take place in the brain and thereby cause severe damage. Since oxidative stress is also observed in stable disease period it could well be that patients experience ongoing neurocognitive damage despite the fact that they do not experience metabolic decompensations. A typical MRI abnormality in MMA and PA patient is the involvement of the basal ganglia, but abnormalities in sulci and fissures as well as delayed myelination are also reported. We recommend to perform brain MRI (MRS) studies in classical OAD patients in order to monitor the patient and gain knowledge on potential other causative factors (such as mitochondrial failure) for disturbed cognitive development.

Within the different UCD subgroups the frequency of impaired cognitive development differs. In 34% to 66% of the patients the cognitive development is impaired [2]. In UCD patients, cognitive outcome mainly depends on the severity and duration of the first hyperammonemic crisis [38]. Within the different UCD subgroups the frequency of impaired cognitive development differs, with 34% to 66% of patients having an impaired cognitive development [2]. In UCD, brain MRI (MRS) studies are recommended to monitor patients, even if they have a normal cognitive development, to correlate anatomical characteristics on brain MRI to cognitive development [2].

1.5 | Conclusions on main outcomes regarding dietary treatment

From this thesis the following recommendations on dietary treatment are essential:

1. For the treatment of UCD and classical OAD patients, use the RDA as a guide when determining protein prescription.
2. Be very aware of the potential harmful consequences of high protein prescription on patient outcome, even when protein prescription is in line with or close to the RDA.

3. In MMA and PA patients be aware of harmful consequences of AAM prescription.
4. Monitor BCAA plasma levels and ratio's closely and frequently in UCD (mainly in those treated with sodiumphenylbutyrate) and in classical OAD patients.
5. In classical OAD patients, measure FGF-21 (and other (potentially newly identified) markers/ material for metabolomics) levels in stable period to guide patients disease progression/improvement.

2 | Long-term (mitochondrial) complications due to improved survival

In MMA and PA patients several long-term complications may occur. Some are due to mitochondrial dysfunction, of which cardiomyopathy, optic atrophy, abnormality of the liver, pancreatitis, pancytopenia, seizures and abnormalities of the basal ganglia are of major importance [39]. In the Dutch MMA and PA cohort mitochondrial complications were present in 70 of the 76 patients, and were mild in 30, moderate in 13, severe in 12 and very severe in 14 patients. Interestingly renal failure is mainly observed in MMA, while cardiomyopathy is mainly observed in PA patients. To date no clear explanations have been found to explain why MMA and PA patients differ in these types of complications. It could be due to the difference metabolites in PA and MMA. In PA 3-hydroxypropionate, methylcitrate, tiglylglycine and propionylgycine as well as glycine are increased, while this is less in MMA. Different tissues have different enzyme activity (MUT and PCC) and different vulnerability towards mitochondrial stress and it could be that specific tissues are more vulnerable to certain metabolites. In UCD, a major complication is liver failure, mainly in OTCD, ASS-D, ASL-D, Arg1, and HHH syndrome patients. In ASL-D arterial hypertension can be observed and should be monitored. In Arg1 spastic paraplegia and developmental delay are often observed. In HHH syndrome spastic paraparesis, and coagulopathy can be observed.

In keeping a balanced scale in the individual patients, energy intake and uptake is important. In classical OAD mitochondrial failure and energy disturbance is a major problem. The study on FGF-21 presented in this thesis gives additional proof to the hypothesis that mitochondrial dysfunction plays a role in long-term complications in classical OAD patients.

Levels of FGF-21 were increased even before mitochondrial complications occurred and we showed that FGF-21 is a good biomarker for (emerging) mitochondrial complications. Manoli et al confirmed our observations on FGF-21[40]. It must be noticed that FGF-21 is influenced by several other factors, other than mitochondrial function. Further research on this biomarker is therefore essential and its role as a potential treatment efficacy measure needs to be established. Mainly with the upcoming newborn screening in these disorders we need biomarkers to determine patient´s disease severity, which may help in establishing and a more personalized treatment approach. It is essential to provide an adequate therapy as early as possible, since mitochondrial failure seems to be an early onset and ongoing process.

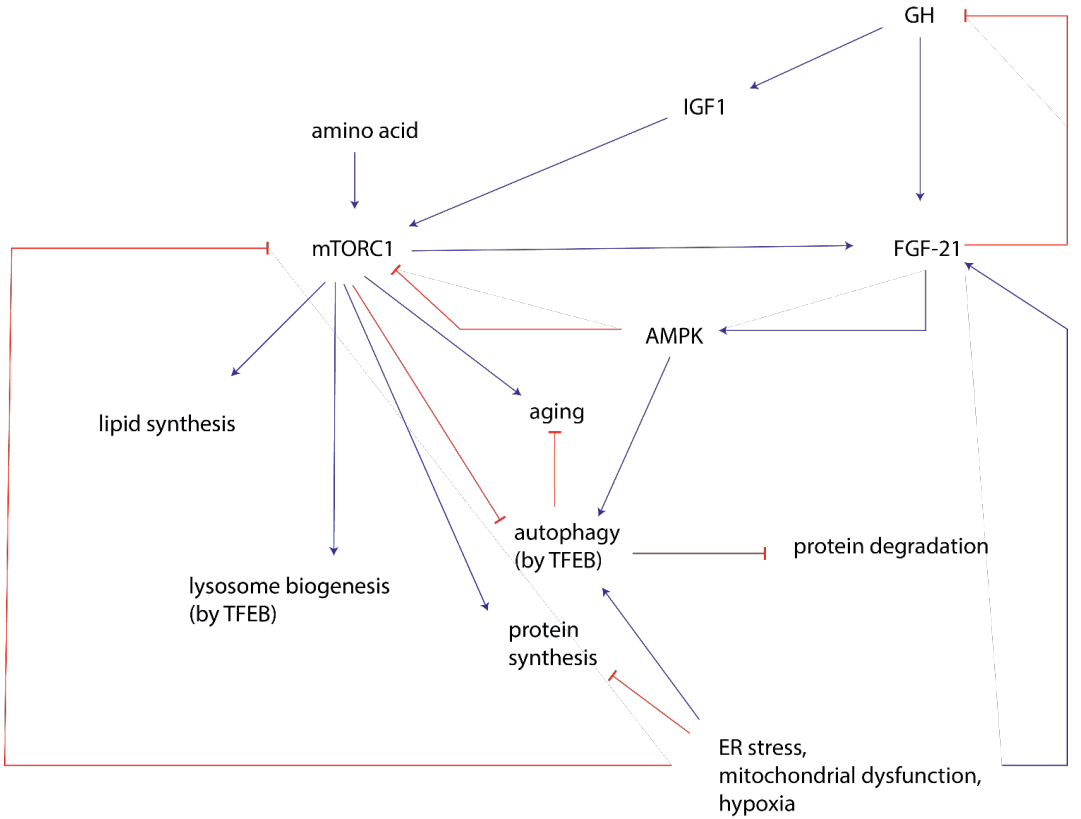
The dysfunction of the mitochondria in classical OAD have been assumed to be due to the direct toxicity of methylmalonic acid. However, in recent publications it is suggested that mitochondrial dysfunction is caused by inhibition of the respiratory chain by 2-methylcitric acid, malonic acid and propionyl-CoA [41]. Factors influencing mitochondrial function besides toxic metabolites in these patients are plasma BCAA levels and L-arginine[17]. The latter two have also been shown in this thesis to play an important role in growth. L-arginine is a precursor for the production of nitric oxide and of creatine [42] and plays a role in improving anti-oxidant defenses [43, 44] and thereby potentially improve disease outcome [45-49].

If we look more in depth into the mitochondria the close interaction between mTORC1, protein regulation and mitochondrial function needs to be taken into account (figure 1). mTORC1 is a protein that plays an important role in cell growth. Its expression is depended on several factors. A higher amino acid availability, mainly by L-leucine and L-arginine, increases mTORC1 expression. Increased mTORC1 increases protein synthesis, aging, lipid synthesis, and mitochondrial function, and it blocks autophagy and lysosome biogenesis [50-53] (figure 1). Furthermore, increased mTORC1 expression increases FGF-21 levels, the biomarker identified in this thesis for disease severity in MMA and PA patients. The positive stimulation of mTORC1 expression on protein expression can be disturbed due to endoplasmic reticulum stress, mitochondrial dysfunction and hypoxia. This will lead to decreased protein synthesis and it will stimulate autophagy. Given that in MMA and PA mitochondrial dysfunction is an ongoing process in the severely affected patients, it is questionable what the effect of the amino acid availability will be on protein synthesis in these patients.

Regarding the role of mTORC1 towards aging, it is interesting that in recent publications and newspaper articles show that a decreased dietary intake can decrease accelerated aging [54] (Volkskrant 21-09-2019). While in UCD and classical OAD patients no direct effect on aging or DNA repair dysfunction is known or shown to date, it is stimulating to look into potential overlaps. And we should question ourselves do we harm the patients by overfeeding and thereby overloading the capacity of the mitochondria. This potential overfeeding has also been observed in intensive care unit patients [55]. MTORC1 gene expression is also influenced by growth hormone. FGF-21 is stimulated by growth hormone, but FGF-21 can also decrease growth hormone production. Growth hormone is applied in a minority of the patients in order to stimulate growth. While we now know that in severely affected patients FGF-21 levels are highly increased it needs to be further studied what the effect of FGF-21 on growth hormone and mTOR plasma levels will be. Interestingly, Sirolimus/rapamicin which can be used as immunosuppressive medication after transplantation, is a known inhibitor of mTOR [56].

The induced mitochondrial long-term complications can be very severe, leading to blindness and even death. While several studies have been performed on the potential efficacy of anti-oxidant treatment in MMA and PA mouse models and patients, to date no clear advices on their use are available [57-60]. Some report beneficial effects, while others report no effects. We suggest that mitochondrial dysfunction is an ongoing process independent of metabolic control. Liver and/or kidney transplantation can potentially prevent the development of long-term mitochondrial complications. It could be that a liver transplant is sufficient to lower the oxidative stress induced by mitochondrial failure and thereby prevent these complications. Liver and/or kidney transplantation seems a safe method in AOA, regarding mortality risk. Survival rate ranged 78% to 100% and complications occurred in approximately 5-20%. However collaboration between European centers is essential, not only to improve surgical techniques, but also to determine how to treat patients after transplantation. Continuation of a protein restricted diet is suggested but not evaluated with regard to long term outcome in MMA and PA patients after transplantation. To date, we cannot yet advise when to start anti-oxidant treatment or when to transplant, we can however advise to monitor FGF-21 levels in all patients and look into the effects of different treatment strategies on plasma FGF-21 levels.

FIGURE 1. Overview of interactions between mTORC1 and other factors. Blue lines indicate positive feedback and red lines indicate negative feedback. Abbreviations: AMPK: AMP-activated protein kinase, ER: endoplasmic reticulum, GH: growth hormone, mTORC1: mammalian target of rapamycin comple 1, IGF-1: insulin-like growth factor 1, TFEB: transcription factor EB.



3 | Recommendations

In the guideline by Baumgartner et al [18] together with information retrieved from our study in the Dutch MMA PA patient cohort we hereby provide a table (Table 1) suggesting what to monitor and a second table (Table 2) when to screen for the different complications. A biobank collecting patient plasma samples is very useful regarding future research questions.

Table 1. Guide to monitor OAD patients

	Items to record	**Diagnostics to perform**
Patient characteristics		
Patient	Day of birth Sex	
Family	Ancestry Consanguinity Length parents	
Mutation	g. / c. / p. coding sequence	Genetic testing
Vitamin B12 responsiveness	Type enzymatic assay and results	Enzymatic assay B12 responsiveness
PCC activity	Type enzymatic assay and results	Enzymatic assay PCC activity
Death	Day of death, Cause of death	
Follow-up	Age at last follow-up	
Adverse outcome of first sympt. phase		
First presentation	Symptomatic/asymptomatic Day of first symptoms Day of diagnosis	
Adverse outcome first presentation	Day of brain MRI Brain MRI results	Brain MRI at set times
	Movement disorder	Consult neurologist
Metabolic instability		
Number of AMD	Day of admission Day of release Reason of admission BCAA levels Protein intake at admission, protein prescription after hospital stay	
Dietary treatment		
	Each time diet changes: Date of change Natural protein intake Amino acid mixture: name, protein intake Single amino acid prescription Kcal prescription Vitamin/mineral supplementation Diet diary Calculate P:E ratio	
Patient follow-up		
	According to the guideline: Physical activity BCAA levels at each clinical visit	

Table 2. When to screen for certain complications in OAD patients

	Items to record	When to screen for	Diagnostics to perform
Cognitive function			
Cognition	Day of neuropsychological tests Neuropsychological test type Neuropsychological test results; IQ	At set times	Neuropsychological tests
Education	School career Type of employment		
Mitochondrial complications			
Hepatomegaly	Day of diagnostic study	From birth onwards, each visit by physical examination	Liver ultrasound at set times
Epilepsy	Results of diagnostic study	At indication	EEG at set times
Cardiomyopathy	Presence complication yes/no	From six years onwards, annually	Cardiac ultrasound at set times
Prolonged QTc interval		From birth onwards, annually	ECG at set times
Optic atrophy		From six years onwards, annually	Consult ophthalmologist at set times
Renal failure	eGFR, plasma creatinine	From one year onwards, every 6 months	Urine kidney function biochemistry at set times
Pancreatitis, anemia, leukopenia, thrombocytopenia, pancytopenia		From birth, every 6 months	Complete blood count at set times
Sensorineural hearing loss		On indication: consult ENT doctor, gastro-enterologist, neurologist, gynecologist, psychiatrist, physical therapist	
Acute psychosis			
Stroke-like episodes			
Premature ovarian insufficiency			
Exercise intolerance			
Muscular hypotonia			
Feeding problems			
Constipation			
Autism			
Attention deficit hyperactive disorder			
Treatment-related complications			
Bone mineral density Growth retardation Obesity	Day of diagnostic study Results of diagnostic study Presence complication yes/no Visit date each visit Weight and length each visit	At set times Each clinical visit	DEXA-scan at set times
Other:			
Keep in mind rarely described complications as pes planovalgus, gout and urolithiasis.			

DEXA: dual-energy X-ray absorptiometry; ECG: electrocardiogram; EEG: electroencephalogram; ENT: ear-nose-throat; MRI: magnetic resonance imaging; PCC: propionyl-CoA carboxylase; IQ: intelligence quotient.

4 | Future perspectives: Sharing data to future research

In this thesis we evaluated current treatment and presented suggestions for the improvement of treatment strategies in classical OAD and UCD.

In MMA and PA: 1) high protein prescription, above 100%RDA, should be avoided since this induces harmful effects, 2) AAM products should be prescribed with care since can induce complications and disturbed plasma BCAA levels, 3) leucine can potentially be used as treatment strategy, but not in such high amounts as in currently applied AAM, 4) growth should not be the main goal of treatment if this necessitates a protein prescription above 100%RDA, and 5) FGF-21 should be used to monitor patients.

In classical OAD, more specific advices on the amount of natural protein prescription within the different severity groups need to be formed based on new long-term follow-up studies. Furthermore, specific indications when to start or stop with AAM products are necessary and new AAM products with lower leucine content seem essential. In future studies the possible harmful consequences (i.e., the number of decompensations, growth, long-term complications and mortality) of the very low plasma BCAA levels in MMA, PA and UCD patients must be evaluated prospectively. Recommendations on adequate BCAA plasma levels in OAD and UCD should be formulated and the efficacy of adjusted treatment (including AAM and/or SAA), without inducing toxicity, needs to be followed. Even more importantly the effect of dietary treatment on the interaction on the LAT transporter needs to be studied. In this also the potential effect of leucine, either or not within AAM (not in such high amounts of currently available), as a treatment to lower valine and isoleucine uptake and thereby increase the possibility to achieve natural protein RDA without complications is essential.

The role of potential biomarkers, FGF-21 as well as other biomarkers for mitochondrial dysfunction, like growth differentiation factor 15 (GDF-15), should be included in order to identify their role and relation with the occurrence of long-term complications in these disorders. Further research should determine if FGF-21 or other mitochondrial markers such as GDF-15, could play a role in the evaluation of preventive strategies concerning the consequences of mitochondrial impairment, and if FGF-21 plays a pathogenic role in the development of mitochondrial impairment. Other biomarker will be likely to be identified by the use of metabolomics.

In UCD a relative high protein intake was prescribed, but low BCAA levels were found and BCAA plasma levels were associated with growth. In UCD further research is necessary to identify the potential harmful effects and shortcomings of the relatively high protein prescription and hereafter guidelines needs to come up with more specific advices on the amount of natural and total protein intake.

Further research should desirably be more focused on personalized medicine. This could for example be achieved by using patient fibroblast and preferable IPS cells to construct cell models (liver, heart and brain) and perform research on these models. Not only the effect of certain amino acids could be studied but also certain medications, such as tacrolimus. Another method could be to determining protein requirement by amino acid oxidation method or nitrogen balance studies in MMA and PA patients [61, 62].

Additional guidelines regarding transplantation should be established on MMA and PA after evaluation of the transplanted patients. In a European wide registry all data regarding transplantation should be studied in detail, including all metabolic data. Regarding transplantation as treatment option cooperation between transplantation centers is required within Europe. It needs to be discussed if we need to work with specific transplantation centers for patients with IMD. Specific transplantation centers can work with more experienced transplantation teams with strong collaboration with metabolic specialists.

The data collected in the Dutch MMA and PA patient cohort can form a good basis for future research. The goal for collecting this data was to study the cohort as a historic control before the introduction of the newborn screening in the Netherlands. The collaboration with the transplantation centers in Europe can be used to perform a more in depth research/questionnaire regarding transplantation in classical OAD.

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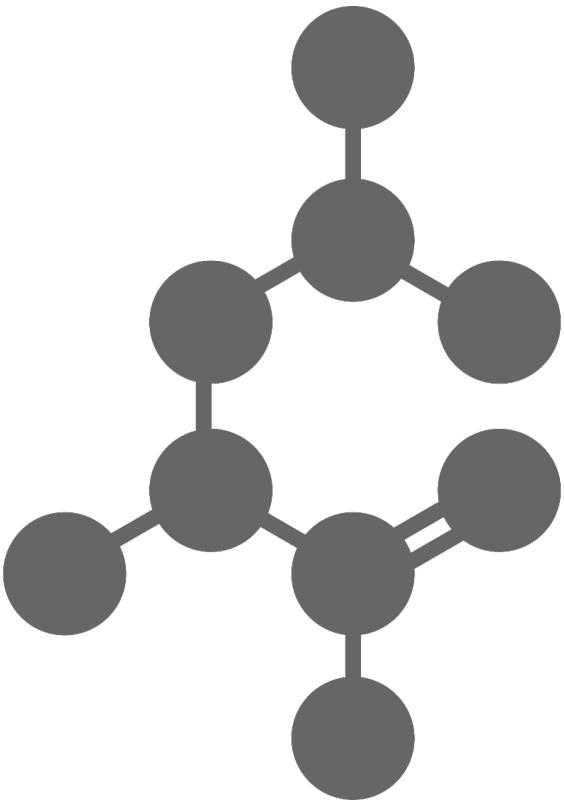

ADDENDUM

Summary

Inborn errors of metabolism are rare disorders but often with severely impaired patient outcome. This thesis focused on the inborn errors in protein metabolism. Within the inborn errors in protein metabolism different disorders can be distinguished, one of these are a group of disorders belonging to the amino and organic acid related disorders (AOA). The main diseases discussed in this thesis are: classical organic acidemias (OAD) and urea-cycle disorders (UCD).

In classical OAD and UCD patient outcome is still unsatisfactory despite the fact that patients have now been diagnosed for several decades. The main aim of this thesis was therefore to define how to improve current available treatment strategies in these disorders. To do so we had four sub-aims:

1. To describe current practice and natural history in classical OAD and UCD patients (chapter **2, 3** and **6**).
2. To describe the consequences of current treatment and how to potentially improve dietary treatment in MMA and PA patients (chapter **2** and **4**).
3. To describe whether to screen for and how to monitor MMA and PA patients (chapter **5** and **6**).
4. To define the safety of liver and/or kidney transplantation in AOA patients as an alternative treatment (chapter **7**).

We identified the shortcomings of current dietary management by evaluating the E-IMD cohort, consisting of 271 OAD and 361 UCD patients, and by long-term retrospective cohort study of 76 Dutch MMA/PA patients. Importantly, in both classical OAD and UCD a high protein intake was prescribed in the majority of the patients. Natural protein prescription exceeded the recommended daily allowances by the WHO in 63% of the classical OAD patients and in >50% of the UCD patients in the E-IMD cohort. In the Dutch MMA/PA cohort natural protein prescription exceeded RDA in 37% and total protein prescription exceeded RDA in 84% of all measured protein prescriptions. A high total protein prescription was often the result of additional amino acid mixtures (AAM) prescription. Despite this high protein prescription, plasma branched chain amino acid (BCAA) levels were low in approximately 55% of the OAD patients and 30% of the UCD patients. Ratios of these BCAA were disturbed, mainly in OAD patients receiving AAM with a L-isoleucine: L-leucine: L-valine ratio of 1.0:3.0:3.2 (normally 1.0:2.0:4.0). A potential cause for the disturbed BCAA levels, despite an appropriate protein prescription in MMA and PA patients, is the high leucine content (due to interaction with other BCAA at the large neutral amino acid transporter (LAT)) in amino acid mixtures prescribed. In UCD patients sodiumphenylbutyrate causes low plasma BCAA levels. In MMA and PA patients, a higher natural protein (mainly when exceeding RDA) as well as a higher AAM prescription

are associated with more frequent severe complications, namely cognitive development delay, mitochondrial complications, and metabolic decompensations. Considering the harmful effects of current applied treatment we need to adjust dietary management in MMA and PA patients.

Growth is disturbed in the majority of OAD and UCD patients. In both OAD and UCD patients (from the E-IMD registry), height was positively associated with the natural protein: energy prescription, and in OAD also negatively with the synthetic protein prescription (a finding that was confirmed in the Dutch MMA/PA cohort). In OAD plasma L-valine and L-arginine levels and in UCD plasma L-valine and L-isoleucine were positively associated with height.

Furthermore, we looked into the potential effect of newborn screening (NBS) in MMA and PA patients based on the long-term retrospective Dutch cohort study in 76 MMA/PA patients. In MMA and PA patients, NBS thus far does not seem to improve outcome regarding metabolic decompensations, and mitochondrial complications. It can potentially have a beneficial effect on the occurrence of a neurological severe adverse outcome after the first presentation with a preliminary estimated reduction of 46% to 36-38% in the occurrence of an adverse outcome. Screening results in the Netherlands will frequently be available after the first presentation in neonatal onset patients (often presenting within the first week after birth).

We identified a biomarker (FGF-21) to identify risk groups on mitochondrial complications established on a national cohort study consisting of 17 classical OAD patients. In MMA/PA patients a median plasma FGF-21 level >1500 Pmol/L, measured during stable disease period, had a positive predictive value of 0.83 and a negative predictive value of 1.00 on long-term mitochondrial complications.

Lastly, since with current dietary treatment outcome seem to remain unsatisfactory we looked into the safety of liver transplantation. Regarding transplantation in the whole group of AOA, transplantation seems save regarding mortality risk with a survival rate at a median of 3.5years follow-up of 78-100%. The mortality risk was the highest within 14 days after transplantation and in PA patients transplanted before they were 4 years of age. Risk on neurological complications mainly in MMA Mut0 patients needs to be taken into account, since calcineurin inhibitors (CNI) can cause neurotoxicity and even a posterior reversible encephalopathy syndrome (PRES) (with an estimated incidence of 9% based on a review of the literature). More specified treatment after transplantation needs to be established.

Samenvatting

Aangeboren stofwisselingsziekten zijn zeldzame aandoeningen, en gaan vaak gepaard met een ernstig ziektebeloop. In dit proefschrift is er onderzoek gedaan naar aangeboren aandoeningen in het eiwitmetabolisme. Binnen deze aandoeningen kunnen verschillende ziektegroepen worden onderscheiden, waarvan de groep aminozuur en organische acidemieën de groep is waar dit proefschrift over gaat. De belangrijkste ziekte categorieën besproken in dit proefschrift zijn: organische acidemieën (OA) en ureumcyclusdefecten (UCD). Binnen de OA, hebben de volgende klassieke OA: methylmalonacidemie (MMA) en propionacidemie (PA) de nadruk.

Patiënten met een klassieke OA of een UCD kunnen al sinds tientallen jaren gediagnosticeerd en behandeld worden. Desondanks hebben patiënten een slechte prognose. Het doel van dit proefschrift was om te bepalen of en hoe we de huidige toegepaste behandeling binnen deze patiëntengroep kunnen verbeteren. Om dit te bepalen zijn er vier subdoelen opgesteld, namelijk:

1. Het beschrijven van de huidig toegepaste behandeling en het natuurlijk beloop van patiënten met een klassieke OA en een UCD (hoofdstuk **2, 3** en **6**).
2. Het beschrijven van de gevolgen van de huidig toegepaste behandeling en hoe we eventueel de dieet behandeling van de patiënten met MMA en PA kunnen verbeteren (hoofdstuk **2** en **4**).
3. Het beschrijven of screenen via de hielprikscreening zinvol is voor MMA en PA en hoe we patiënten met deze aandoeningen kunnen monitoren (hoofdstuk **5** en **6**).
4. Het beschrijven van de veiligheid van lever en/of niertransplantatie als alternatieve behandeling in patiënten met een aminozuur en/of organische acidemie (hoofdstuk **7**).

In dit proefschrift hebben we laten zien hoe we in de huidige dieet behandeling van patiënten tekortschieten door middel van een evaluatie van 271 OA en 361 UCD patiënten uit de E-IMD database en door middel van een retrospectieve cohortstudie van 76 Nederlandse MMA en PA patiënten. In zowel patiënten met een OA als in patiënten met een UCD werd een hoge hoeveelheid eiwit voorgeschreven. Het natuurlijk eiwit voorschrift was

hoger dan de aanbevolen dagelijkse hoeveelheid door de WHO in 63% van de klassieke OA en >50% van de UCD patiënten uit de E-IMD database. In het Nederlandse MMA/PA cohort was het voorschrift van het natuurlijk eiwit hoger dan de aanbevelingen in 37% van alle momenten dat er eiwitvoorschrift was beschreven en van het totaal eiwit in 84%. Echter ondanks deze hoge inname van eiwit waren de plasma spiegels van de vertakte keten aminozuren laag in 55% van de OA en in 30% van de UCD patiënten (E-IMD cohort). De verhoudingen van deze vertakte keten aminozuren waren verstoord, met name in OA patiënten die aminozuurproducten gebruikten. Deze laatste groep patiënten had een ratio van 1.0:3.0: 3.2 van L-isoleucine: L-leucine: L-valine, welke normaal 1.0:2.0:4.0 is. In MMA en PA patiënten kunnen deze lage/verstoorde plasma spiegels het gevolg zijn van een hoge hoeveelheid leucine (door interactie met andere vertakte keten aminozuren op het LAT transporteiwit) in de aminozuurproducten die vele patiënten gebruiken. In UCD worden de lage plasma vertakte keten aminozuren vaak veroorzaakt door het gebruik van sodium phenylbutyrate. In MMA en PA patiënten was een hoge inname van natuurlijk eiwit als wel een hoge inname van aminozuurmengsels geassocieerd met een slechte prognose, namelijk met het optreden van ontwikkelingsbeperking, mitochondriële complicaties (energiewinning complicaties) en metabole ontregelingen. Gezien deze schadelijke gevolgen van de huidige hoeveelheid voorgeschreven eiwit is het essentieel om de behandeling van MMA en PA aan te passen.

De lengtegroei was in de meerderheid van de OA en UCD patiënten verstoord. In beide ziektebeelden was een hoger voorschrift natuurlijk eiwit: energie geassocieerd met een positieve lengtegroei. In OA had een hoger voorschrift van aminozuurproducten een negatieve associatie met lengtegroei (dit werd ook gevonden in het Nederlandse sub-cohort). In OA waren de plasma spiegels van L-valine en L-arginine en in UCD de plasma spiegels van L-valine en L-isoleucine geassocieerd met positieve lengtegroei.

Daarnaast hebben we gekeken naar de mogelijke positieve effecten van het toevoegen van MMA en PA aan de hielprikscreening in Nederland gebaseerd op een retrospectieve cohortstudie van 76 MMA/PA patiënten. Op basis van onze studie is te concluderen dat op dit moment er geen positief effect te verwachten valt van deze hielprikscreening op het aantal metabole decompensaties en mitochondriële complicaties in MMA en PA patiënten. Het is mogelijk dat in enkele patiënten de ziekte ontdekt wordt voordat een ernstige eerste

decompensatie optreedt en er kan daarbij een verwachte afname zijn van 48% naar 36-38% in het optreden van neurologische schade bij de eerste klinische presentatie. Echter zijn er veel patiënten die zich op zeer jonge leeftijd al presenteren (binnen de eerste levensweek), voordat er uit de hielprikscreening bekend is of ze MMA of PA hebben. In dit proefschrift tonen we een mogelijke biomarker, namelijk FGF-21, die gebruikt kan worden om risicogroepen op mitochondriële complicaties te identificeren. Deze studie werd uitgevoerd bij 17 klassieke OA patiënten. Een mediane FGF-21 plasma spiegel van >1500 Pmol/L, gemeten in stabiele periode, had een positief voorspellende waarde van 0.83 en een negatieve voorspellende waarde van 1.00 op het optreden van mitochondriële lange-termijn complicaties.

Omdat de huidige behandeling met een dieet niet afdoende is (en momenteel het enige alternatief) om te zorgen voor een mogelijke verbetering van de prognose in patiënten met een OA of UCD hebben we gekeken naar de veiligheid van het toepassen van lever en/of niertransplantatie. Gezien de relatief lage mortaliteit gevonden in onze studie (78-100% overleving bij een gemiddelde follow-up van 3.5 jaar) kunnen we concluderen dat transplantatie een veilig alternatief is. Het overlijdens risico was het hoogst binnen 14 dagen na de transplantatie en bij PA patiënten die getransplanteerd werden op een leeftijd jonger dan 4 jaar. Er zijn veel patiënten met complicaties na transplantatie, waarin we bij de MMA mutase negatieve patiënten met name vaak neurologische complicaties zagen. Calcineurine-remmers zijn immunosuppressiva die neurologische schade en zelfs een posterior reversible encephalopathy syndrome (PRES) kunnen veroorzaken. De incidentie van PRES in MMA patiënten na transplantatie werd geschat op 9% op basis van een literatuur review van de getransplanteerde MMA patiënten. Het is belangrijk om ook deze uitkomsten mee te nemen en goed te definiëren hoe we patiënten na een transplantatie behandelen.

Dankwoord

Mijn promotietraject heb ik met heel veel plezier kunnen afronden dankzij jullie allemaal. Maar bovenal wil ik alle patiënten en ouders die hebben willen meewerken aan de verschillende onderzoeken/databases bedanken. Ik hoop van harte dat met dit promotietraject we de zorg voor de patiënten weer een stapje vooruit hebben kunnen helpen.

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nieuwe mensen gezien en een hoop tafeldansen gedaan. Marein jou wil ik echt enorm bedanken, je was er altijd voor me en zag direct als ik niet goed in mijn vel zat. Door met jou te sparren kon ik weer stapjes vooruit en heb ik me kunnen ontwikkelen tot wie ik nu ben. Ik zal je vast af en toe bellen om even mijn hart te luchten en er samen om te kunnen lachen. Ik hoop van harte dat je je plekje en je rust gaat vinden, je bent een topper. Chris wat jammer dat we maar kort samen op de kamer hebben gezeten, maar dank dat ik je plek mocht overnemen, lekker achter mijn schermen in de hoek. R heeft helaas weinig open gestaan. Jeroen, ik heb genoten van je humor, de verwarming blijft een ding, maar dat is alleen maar mooi. Imke, ik vond het fijn om met jou ook de lastige kanten te kunnen bespreken, ik vind het erg knap hoe je hebt doorgezet en hoop dat je uiteindelijk je draai vindt. Kom gerust eens langs op mijn nieuwe werkplek, hebben we vast enorme lol. Emma, ik vind het mooi om te zien hoe je je weg hebt gevonden op de kamer en hoop dat je heel veel plezier zal beleven aan de komende tijd. Meng, je bent echt een topper en dank voor de heerlijke lachbuien. Wilma, dank voor de pepermuntjes, en natuurlijk je hulp, kritisch blik en adviezen, jij blijft gewoon volhouden maar vergeet af en toe jezelf niet.

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De club8, natuurlijk niet te vergeten. Niet een club8, nee de club8, ja die ja. Dank mannen, dat we onze reünietjes blijven behouden. Fem, Fem en Ol dank dat jullie mijn lieve vriendinnen zijn. Pien, we zien elkaar niet zo vaak, maar dank voor je lieve steun van afgelopen jaren.

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About the author

Femke Molema was born on the 5th of October 1989, in Dordrecht, the Netherlands. In 2008 she graduated the gymnasium. She decided to study medicine in Utrecht, which she graduated from in 2016. During these studies she was already inspired to do research in the metabolic field. While studying medicine at the university she enthusiastically coached different rowing teams.

In 2016 she started a one year research project on arginine and height in organic acidemias and urea cycle disorders in the Erasmus MC. Thereafter she went to Surinam to gain clinical knowledge while working as a resident (ANIOS) in the pediatrics department. After moving back to the Netherlands in 2018 she continued her research work in the Erasmus MC for another 2 year and finished her PhD project under supervision of Monique Williams and Ans van der Ploeg.

List of publications

Evaluation of dietary treatment and amino acid supplementation in organic acidurias and urea-cycle disorders: On the basis of information from a European multicenter registry.
Molema F, Gleich F, Burgard P, van der Ploeg AT, Summar ML, Chapman KA, Barić I, Lund AM, Kölker S, Williams M; Additional individual contributors from E-IMD.
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J Inherit Metab Dis. 2018 Nov;41(6):1179-1187. doi: 10.1007/s10545-018-0244-6. Epub 2018 Aug 29. PMID:30159853

Retrospective evaluation of the Dutch pre-newborn screening cohort for propionic acidemia and isolated methylmalonic acidemia: what to aim, expect and evaluate from newborn screening?
H.A. Haijes[a], **F. Molema[a]**, M. Langeveld, M.C. Janssen, A.M. Bosch, F.J. van Spronsen, M.F. Mulder, N.M. Verhoeven-Duif, J.J.M. Jans, A.T. van der Ploeg, M.A. Wagenmakers, M.E. Rubio-Gozalbo, M.C.G.J. Brouwers, M.C. de Vries, J.G. Langendonk, M. Williams[b], P.M. van Hasselt[b] [a]These authors contributed equally [b] These authors contributed equally. J Inherit Metab Dis. 2019 Dec 11. doi: 10.1002/jimd.12193 [Epub ahead of print]

Neurotoxicity including PRES after initiation of calcineurin inhibitors in transplanted methylmalonic acidemia patients: two case reports and review of the literature.
Femke Molema, Monique Williams, Janneke Langendonk, Sarwa Darwish-Murad, Jacqueline van de Wetering, Ed Jacobs, Willem Onkenhout, Esther Brusse, Anke W van der Eerden, Margreet Wagenmakers. J Inherit Metab Dis reports 2020.

High protein prescription in methylmalonic and propionic acidemia patients and its negative association with long-term outcome**.**
F. Molema[a], H.A. Haijes [a], M.C. Janssen, A.M. Bosch, F.J. van Spronsen, M.F. Mulder, N.M. Verhoeven-Duif, J.J.M. Jans, A.T. van der Ploeg, M.E. Rubio-Gozalbo, M.C.G.J. Brouwers, M.C. de Vries, M.A. Wagenmakers, P.M. van Hasselt[b], M. Williams[b]. [a]These authors contributed equally [b] These authors contributed equally. Submitted.

Liver and/or kidney transplantation in amino and organic acid related disorders. European practices and outcome.
F. Molema, D. Martinelli, F. Horster, S. Kolker, T. Tangeraas, B. de Koning, C. Dionisi Vici , M. Williams, additional individual contributors from the SSIEM. Submitted.

PhD portfolio

	Year	Workload (ECTs)
General courses		
- Basiscursus Regelgeving Klinisch onderzoek (BROK)	2017	1.0
- Integrity in Research	2017	0.3
- Endnote	2017	0.14
- Open Clinica	2017	0.14
- R basic	2019	1.8
- Englisch biomedical writing and communication	2019	2.0
Research skills		
- Introduction to data analysis	2016	0.7
- Principles of research in medicine and epidemiology	2016	0.7
- Biweekly Research Meeting, Center for Lysosomal and Metabolic Disease	2016-2020	2.0
Presentations and (Inter)national conferences		
- Society of Inborn Errors of Metabolism (SSIEM), Rome, Italy (poster presentation)	2016	1.0
- EIMD meeting, Rome, Italy	2016	0.2
- Sophia Research Day, Rotterdam, the Netherlands	2017	0.3
- Erfelijke Stofwisselingsziekten Nederland (ESN) Voorjaars Symposium, Leuven, Belgium (oral presentation)	2017	1.0
- EMG meeting, Zagreb, Croatia (oral presentation)	2018	1.5
- Patientendag Organische Acidemieen, Netherlands (oral presentation)	2018	0.7
- Erfelijke Stofwisselingsziekten Nederland (ESN) Najaars Symposium, Utrecht, Netherlands (oral presentation)	2018	0.7
- Patientendag Non Ketotische Hyperglycemie (oral presentation)	2018	0.3
- EIMD meeting, Brussels, Belgium (oral presentation)	2018	2.0
- Society of Inborn Errors of Metabolism (SSIEM), Athens, Greece (poster presentation)	2018	1.0
- Dietistendag (Organische Acidemieen) Nutricia (oral presentation)	2019	0.5
- Grand round Sophia Erasmus MC (oral presentation)	2019	0.5
- E-IMD meeting	2019	

-	Sophia research day (oral presentation)	2019	0.5
-	EMG conference 2019 Lyon	2019	0.1
-	SSIEM, Rotterdam	2019	1.0
-	AOA subnetwork meeting Frankfurt (oral presentation)	2019	1.0
		2019	2.0
-	Erfelijke Stofwisselingsziekten Nederland (ESN) voorjaarssymposium, Doorn, Netherlands (oral presentation)		
-	Erfelijke Stofwisselingsziekten Nederland (ESN) najaarssymposium, Utrecht, Netherlands (oral presentation)	2019	0.5
-	AOA meeting Brussels (oral presentation)	2019	0.5
-	MMA and PA guideline meeting Brussels	2019	0.7
		2019	0.7
Other			
-	Succesful scientific writing by Janice R Matthews and Robert W Matthews	2017	1.0
Total			26.5

Abbreviations

AAM(s)	Amino acid mixture(s)
AAM(s)-OAD	Amino acid mixture(s) for organic acidemias (lack L-isoleucine and L-valine)
AAM(s)-UCD	Amino acid mixture(s) for urea-cycle disorders (contain essential amino acids)
AMD	Acute metabolic decompensation
AMPK	AMP-activated protein kinase
AO	Adverse outcome
AOA	Amino and organic acid related disorders
OAD	Organic acidemia
ASL (-D)	Argininosuccinate lyase (deficiency)
ASS (-D)	Argininosuccinate synthetase (deficiency)
Arg1	Arginase deficiency 1
BCAA	Branched-chain amino acids
BMD	Bone mineral density
Cbl	Cobalamin
CNI	Calcineurin inhibitor
CPS1 (-D)	Carbamylphosphate synthetase 1 (deficiency)
CSF	Cerebrospinal fluid
DWI	Diffusion-weighted imaging
EO	Early onset
E-IMD	European registry and network for intoxication type metabolic diseases
ER	Endoplasmic reticulum
FGF-21	Fibroblast growth factor 21
FS	Family screening
GDF-15	Growth differentiation factor 15
GH	Growth hormone
HHH	Hyperornithinemia-hyperammonemia-homocitrullinuria
ICU	Intensive care unit
IGF	Insulin-like growth factor

IVA	Isovaleric acidemia
kcal	Kilocalories
LKT	Liver and/or kidney transplantation
LO	Late onset
MMA	Methylmalonic acidemia/aciduria
mma	Methylmalonic acid
MELD	Model for end-stage liver disease
MMF	Mycophenolate mofetil
MRI	Magnetic resonance imaging
MSUD	Maple syrup urine disorder
mTOR	Mammalian target of rapamycin
MUT	Methylmalonyl-CoA mutase
NAGS (-D)	N-acetylglutamate synthetase (deficiency)
NBS	Newborn screening
OAD	Organic acidurias
OTC (-D)	Ornithine transcarbamylase (deficiency)
ORNT	Ornithine transporter
PA	Propionic acidemia/aciduria
P:E ratio	Protein-to-energy ratio (natural P:E ratio is natural protein-to-energy ratio and total P:E ratio is total protein-to-energy ratio)
PCC	Propionyl-CoA carboxylase
POD	Post-operative day
PRES	Posterior reversible encephalopathy syndrome
PY	Patient year
RDA	Recommended daily allowance
r_τ	Kendall's tau rank correlation
SAA	Single amino acids (L-valine and/or L-isoleucine supplied as a supplement, either independently or in combination)
SDS	Standard deviation score
TCA cycle	Tricarboxylic acid cycle (= citric acid cycle)
TFEB	Transcription factor EB
Tyr1	Tyrosinemia type 1

UCD	Urea-cycle disorders
UNOS	United network for organ sharing
WHO	World Health Organization
Ws	Wilcoxon rank sum test