Title

Argininosuccinic aciduria: recent pathophysiological insights and therapeutic prospects

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ABSTRACT

The first patients affected by argininosuccinic aciduria (ASA) were reported 60 years ago. The clinical presentation was initially described as similar to other urea cycle defects, but increasing evidence has shown overtime an atypical systemic phenotype with a paradoxical observation, i.e. a higher rate of neurological complications contrasting with a lower rate of hyperammonaemic episodes. The disappointing long-term clinical outcomes of many of the patients have challenged the current standard of care and therapeutic strategy, which aims to normalise plasma ammonia and arginine levels. Interrogations have raised about the benefit of newborn screening or liver transplantation on the neurological phenotype. Over the last decade, novel discoveries enabled by the generation of new transgenic argininosuccinate lyase (ASL)-deficient mouse models have been achieved, such as, a better understanding of ASL and its close interaction with nitric oxide metabolism, ASL physiological role outside the liver, and the pathophysiological role of oxidative/nitrosative stress or excessive arginine treatment. Here, we present a collaborative review, which highlights these recent discoveries and novel emerging concepts about ASL role in human physiology, ASA clinical phenotype and geographic prevalence, limits of current standard of care and newborn screening, pathophysiology of the disease, and emerging novel therapies. We propose recommendations for monitoring of ASA patients. Ongoing research aims to better understand the underlying pathogenic mechanisms of the systemic disease to design novel therapies.

MANUSCRIPT

Introduction

Sixty years ago, argininosuccinic aciduria (ASA) (OMIM 207900) was first reported with 2 siblings, who presented "*a disease, probably hereditary, characterised by a severe mental deficiency and a constant gross abnormality in amino acid metabolism*" with excretion of "*an unusual urinary amino acid*" (Allan et al 1958), later characterised as argininosuccinate (Westall 1960). ASA is caused by deficient function of argininosuccinate lyase (ASL), which catalyses the transformation of argininosuccinate into arginine, an essential reaction for the waste of excessive nitrogen through the urea cycle and endogenous arginine synthesis. ASA phenotype has overtime been recognised as more complex than other urea cycle defects (UCD) with a systemic phenotype. Here, we present an update on the recent scientific insights and clinical findings in ASA. Ongoing research is progressively deciphering the complex role of ASL in physiology and subsequent pathophysiology of ASA, aiming to identify novel targets for therapy.

1. ASL role in human physiology

ASL transforms argininosuccinate into arginine and fumarate. This cytosolic reaction belongs to 2 metabolic pathways, the urea cycle responsible for detoxifying ammonia into urea, and the citrulline-NO cycle, which synthesises nitric oxide (NO) from arginine via NO synthase (NOS) (**Figure 1**). ASL, although mainly expressed in the liver, is found in various other tissues e.g. skin, hematopoietic system, muscle, heart, kidney, small intestine and brain (Ratner 1973; Nagamani et al 2012). The enzyme is a homotetramer with 4 enzymatic sites (Turner et al 1997). ASL is encoded by the human argininosuccinate lyase gene (*hASL*) located in chromosome 7 (7q11.21) (O'Brien et al 1986) and contains 17,554 base pairs (bp) divided in 16 exons (Balmer et al 2014). An exon 0 has been described coding for the 5' untranslated region (Saudubray et al) (Trevisson et al 2007). *ASL* is a highly conserved gene identified in various species, *e.g.* bacteria, yeast (Crosas et al 2015), vegetables (Xia et al 2014), birds (Sampaleanu et al 2002) and mammals.

In humans, arginine supply is provided by exogenous nutritional intake and ASL-dependent endogenous synthesis, which mainly occurs in the kidney (Nagamani et al 2012) (**Figure 2**). Of note, the liver urea cycle does not contribute arginine to the circulating pool. Arginine is a semi-essential amino acid as endogenous production is sufficient to meet physiological requirement. In different catabolic states as inflammation, or in conditions involving kidney/small intestine dysfunction, arginine becomes an essential amino acids. In ASA, as arginine synthesis is impaired, even after liver transplantation, arginine is essential as the renal production of arginine remains deficient (Rabier et al 1991).

The "arginine paradox" illustrates the observation that despite saturating intracellular arginine levels, supplementation of exogenous arginine can increase the NOS-dependent NO production (Kurz and Harrison 1997; Vukosavljevic et al 2006). This suggests an intracellular compartmentalisation or channelling effect of arginine to reach the active site of NOS. However, this is not observed in ASA where a systemic NO deficiency persists despite exogenous arginine supplementation. This was elucidated by the discovery of the structural role of ASL in maintaining a multiprotein complex including the cationic amino acid transporter CAT1, ASS1, ASL and nitric oxide synthase (NOS) (Li et al 2005; Erez et al 2011). ASL has both a catalytic function enabling arginine production and a structural role in maintaining this protein complex, which channels arginine to NOS via CAT1 for NO production.

Finally, arginine is a precursor for various metabolic pathways underlining the importance of ASL, its ubiquitous expression and its high conservation through evolution (**Figure 1**).

2. Clinical phenotype

Prevalence

ASA is usually considered as the second most common UCD after ornithine transcarbamylase (Inagaki et al) deficiency, accounting for 16% of all UCDs (Summar et al 2013). Recent publications have highlighted high variation of ASA prevalence between countries (**Table 1**).
Patients can present either with an early neonatal-onset (<28 days of age) hyperammonaemic coma, or with a broad late-onset phenotypic spectrum from hyperammonaemic crisis to a

chronic phenotype with neurocognitive, gastrointestinal and liver symptoms without hyperammonaemia (Nagamani et al 2012).

Biochemical presentation/ diagnosis

***Systemic disease (*Figure 3**)

Liver. Liver symptoms reported in 50% of patients are frequent in early-onset patients (Baruteau et al 2017). Hepatomegaly and/or elevated transaminases are the most common signs (Parsons et al 1987; Kleijer et al 2002) and yet some patients present with hyper-ammonaemia during the first few days of life. Liver failure with mild chronic impairment of the liver synthetic function has been reported (Bawle and Warrier 1991; Marble et al 2008). Fibrosis (Zimmermann et al 1986; Mori et al 2002) and cirrhosis (Marble et al 2008) can have fatal consequences (Mercimek-Mahmutoglu et al 2010). Hepatocellular carcinoma has been reported, even in paediatrics (Baruteau et al 2017). The progression of the hepatopathy is independent of ammonia control and can worsen despite adequate treatment (Mori et al 2002).

Central nervous system. A high rate (> 90%) of neurological symptoms is observed in both early- (i.e. patients symptomatic ≤ 28 days of life) and late-onset phenotypes (Baruteau et al 2017). Neurocognitive deficit covers a broad spectrum of severity from borderline IQ (Kleijer et al 2002) to severe mental retardation (Lagas and Ruokonen 1991; Ficicioglu et al 2009). Various subsets of neurodevelopment are affected: gross and fine motor delays, speech delay, learning and memory. Abnormal neurodevelopment is usually diagnosed around 24 months of age (Gerrits et al 1993; Kleijer et al 2002; Grioni et al 2011; Baruteau et al 2017).

Epilepsy, observed in 40% of patients (Baruteau et al 2017), presents with tonic clonic, clonic or myoclonic seizures (Grioni et al 2011). Electroencephalography can be abnormal even in non-symptomatic patients, displaying an aspecific pattern (Verma et al 1984; Grioni et al 2011). Hyperammonaemia-related neonatal seizures can be subclinical (Wiwattanadittakul et al 2018) but are not predictive of developing an epilepsy later in life (Grioni et al 2011).

Global muscular weakness and cerebellar signs (ataxia, tremor, dystonia, dysphagia) have been reported (Lagas and Ruokonen 1991; Baruteau et al 2017).

Behavioural difficulties are noticed from hyperactivity (Lagas and Ruokonen 1991; Kleijer et al 2002), autoaggression with self-mutilation (Sijens et al 2006), autism (Grioni et al 2011) to psychiatric presentations with paranoia (Lagas and Ruokonen 1991), psychosis (Odent et al 1989) or schizophrenia (von Wendt et al 1982).

Brain imaging can display global atrophy, bilateral microcystic periventricular leukomalacia (Grioni et al 2011), basal ganglia T2 hyperintensity, white matter hyperintensities, focal infarct, heterotopia (Lagas and Ruokonen 1991; Baruteau et al 2017). Atrophy and white matter changes have been reported despite normal ammonia levels (Lagas and Ruokonen 1991). Cerebral proton magnetic resonance spectroscopy (1H-MRS) in treated patients with ASA has shown contradictory results with either increased (van Spronsen et al 2006; Sijens et al 2006) or decreased (Roze et al 2007) brain guanidinoacetate and creatine contents in both white and grey matter. Baruteau et al reported decreased creatine and increased guanidinoacetate in white matter (Baruteau et al 2017). L-arginine supplementation has been used to correct low systemic creatine levels observed in urea cycle defects (Arias et al 2004) and this was suspected of causing elevation of brain guanidinoacetate levels (Sijens et al 2006).

Cardiovascular system: High blood pressure has been observed in both early- or late-onset phenotypes with a low prevalence although this might be under-diagnosed (Kolker et al 2015). There is no correlation between age of onset and severity (Brunetti-Pierri et al 2009; Nagamani et al 2012). *Arrhythmias* with either atrioventricular block caused by increased vagal tone (Ozcan et al 2015) or atrial flutter (Baruteau et al 2017) have been reported. *Thrombocytosis* has been noted in early-onset patients from Saudi Arabia (AlTassan et al 2018).

Kidney. Electrolyte disturbances with transient or chronic hypokalaemia (Nagamani et al 2012) and mild chronic renal failure (Kolker et al 2015) have been reported. Hypokalaemia is more frequently observed in early-onset patients (Baruteau et al 2017). Nephrolithiasis has been described (Reid et al 2009).

Gastrointestinal symptoms like protein aversion, poor appetite, recurrent vomiting are common signs in UCDs (Gardeitchik et al 2012). Profuse diarrhoea with aspecific inflammation of gastric and intestinal mucosae and chronic pancreatitis have been described (Ibarra-Gonzalez et al 2010; Baruteau et al 2017).

Hair and skin signs (*trichorrhexis nodosa*, *monilethrix* and *pili torti)* are observed in untreated patients (Coulter et al 1982). Children present with brittle, dry and short brush-like hair, sometimes not requiring any hair cut for years (Hambraeus et al 1974; Schutgens RBH 1979).

These symptoms respond well to arginine supplementation, which is likely explained by the high hair arginine content (>10%) (Nagamani et al 2012); hence the name of "aminogenic" or "arginine-responsive" alopecia (Shelley WB 1965). Severe *dermatitis* of the face and genital areas (Kleijer et al 2002) or arginine-responsive "dry, scaly skin" (Widhalm et al 1992) have been mentioned. Increased frequency in *dental caries* (Hambraeus et al 1974) could be caused by defective immunity with loss of NO antimicrobicidal effect. *Elevated triglycerides* have been found in some patients (personal communication C. Dionisi-Vici). *Asymptomatic* patients have been reported (Ruegger et al 2014).

Genotype-phenotype correlation

Around 140 *hASL* mutations have been reported (Balmer et al 2014), including three mutations with a founder effect: (c.1060C>T; p.GlN354* and c.346C>T; p.GlN116*) in Saudi Arabia and (c.1153C>T; p.Arg385Cys) in the Finnish population, the later associated with high residual activity (Nagamani et al 2012).

There is some genotype-phenotype correlation known with significant residual levels of ASL activity in most of the *ASL* mutations that are associated with a variant clinical and biochemical ASA phenotype (Hu et al 2015). However, also discrepancies exist between ASL activity and phenotype, for example in reports of asymptomatic patients with undetectable ASL activity or in patients with higher residual activity and severe neurocognitive impairment (Mercimek-Mahmutoglu et al 2010). This could be caused by intra-allelic complementation between specific mutations (McInnes et al 1984; Walker et al 1997; Trevisson et al 2007), instability of the ASL protein (Linnebank et al 2000), and/or mutations affecting preferentially the catalytic or the structural function of ASL (Erez et al 2011).

3. Limitation of current standard of care

Poor long-term neurological outcome under conventional treatment

ASA long-term neurological outcome is a paradox. In contrast to proximal UCDs, this outcome is not readily correlated with hyperammonaemia. Among UCDs, ASA patients have a higher rate of neurocognitive deficits (Ruegger et al 2014; Waisbren et al 2016) despite experiencing reduced frequency of hyperammonaemia. Up to 50% of ASA patients with late-onset phenotype have normal ammonaemia at diagnosis and 20% of them do not need any

protein-restricted diet or nitrogen scavengers (Baruteau et al 2017). Patients with either i) late-onset normo-ammonaemic presentation or ii) treated perinatally due to a familial history before hyperammonaemic decompensation, display similar long-term neurological outcome compared to early- or late-onset hyperammonaemic patients (Baruteau et al 2017). This suggests that current therapeutic guidelines, which rely on protein-restricted diet, oral nitrogen scavengers and arginine supplementation (Haberle et al 2012) and primarily aim to normalise plasma ammonia and arginine levels, are not suitable to fully protect the brain from the neurological disease.

Liver transplantation: what benefit?

As in other UCDs, liver transplantation (LT) has been performed in ASA to reduce the risk for hyperammonaemia. 32 ASA patients have so far received LT (Robberecht et al 2006; Marble et al 2008; Newnham et al 2008; Ozcay et al 2015; Yankol et al 2016; Kido et al 2017; Szymanska et al 2017; AlTassan et al 2018; Waisbren et al 2018) (**e-Table 1**). The evaluation of the post-transplant outcome however is fragmentary and mainly focused on surgical and LT-related complications. LT normalizes ureagenesis as shown by normal ammonaemia, discontinuation of protein restricted diet and nitrogen scavengers. Arginine therapy remains necessary for few patients (**Table 2**). Reports claim post-transplant stabilization of neurologic impairment, but no neurocognitive and behavioral assessments are available (Robberecht et al 2006; Marble et al 2008; Newnham et al 2008; Ozcay et al 2015; Yankol et al 2016; AlTassan et al 2018). Plasma argininosuccinate and citrulline levels have been published in one patient only, showing a significant reduction after LT (Marble et al 2008). An ongoing study showed that LT does not reduce argininosuccinate levels in cerebrospinal fluid, contrasting with plasma levels (Ranucci et al 2018). This implies that the metabolic alteration may persist in the brain beyond LT. Thus, LT decision in ASA needs to be well balanced. Currently, its main indications are a poor metabolic control with recurrent hyperammonaemia as a risk for neurotoxicity or chronic liver disease leading to hepatic failure.

Newborn screening: is it worth doing?

Newborn screening for ASA has been reported either by measurement of plasma citrulline (Naylor 1981; Burgard et al 2012; Nagamani et al 2012), blood or urinary argininosuccinate (Wilcken et al 1980; Auray-Blais et al 2007; Ficicioglu et al 2009) or an enzyme-auxotroph test in dried blood spots (Widhalm et al 1992; Mercimek-Mahmutoglu et al 2010). ASA

patients diagnosed by newborn screening in North America and Europe represent 37% and 13% of patients' cohorts, respectively (Posset et al 2018). Interestingly 25% of ASA patients are asymptomatic in North America, where ASA is included in the newborn screening programme versus only 5% in Europe (Posset et al 2018).

The neurological outcome of the patients diagnosed by population-wide newborn screening is significantly better compared with outcome from patients presenting symptomatically with a delayed diagnosis (Widhalm et al 1992; Ficicioglu et al 2009; Mercimek-Mahmutoglu et al 2010) or following a familial index case (Baruteau et al 2017) (**Table 3**). Some of the ASA patients diagnosed by newborn screening show high residual ASL activity (Ficicioglu et al 2009), no episode of hyperammonaemia (Widhalm et al 1992; Ficicioglu et al 2009) even after a protein load (Mercimek-Mahmutoglu et al 2010), normal arginine levels (Ficicioglu et al 2009) or negligible urinary excretion of argininosuccinate (Mercimek-Mahmutoglu et al 2010). Newborn screening might diagnose asymptomatic patients or some with milder phenotype, who will not have been diagnosed otherwise (Ficicioglu et al 2009). Therefore this is a strong bias, which prevents to draw reliable conclusions that an early treatment modifies the neurological phenotype. A recent publication compared the long-term neurological outcome of 10 familial cases diagnosed and treated neonatally to their respective familial proband diagnosed symptomatically. This enabled to compare the outcome between assumed identical genotype with known severity from the index case and to rule out a potential bias from newborn screening. The follow-up did not show a neurological benefit of an early therapeutic intervention with conventional treatment (Baruteau et al 2017), suggesting that an early therapeutic intervention with current standard of care does not protect against the ASA-related neurological disease.

4. Pathophysiology

Although the ASA pathophysiology remains partially elusive, various mechanisms have been hypothesized to play a role in the disease: hyperammonaemia, deficiency of arginine and downstream metabolites as NO, toxicity of argininosuccinate and conjugated metabolites and NO related oxidative stress **(Figure 4)**.

Hyperammonaemia

There is a vast literature on the consequences of elevated ammonia and the reader is referred to the following literature (Bachmann 2002; Monfort et al 2005; Braissant et al 2013) (Guertin et al 1983; Bergeron et al 1990; Rangroo Thrane et al 2013).
Argininosuccinate, which contains two nitrogen moieties, enables a partial waste of nitrogen, which reduces ammonia accumulation (Nagamani et al 2012).

Arginine and downstream metabolites' deficiency

Arginine supplementation can enhance synthesis and excretion of substrates of argininosuccinate synthetase (ASS1) and ASL, hereby increasing urinary waste nitrogen loss (Batshaw et al 1982; Braissant et al 2002). This was the basis to add arginine in the management of hyperammonaemia, both in the acute setting as well as long-term. In a study of four patients, arginine was found to be an indispensable amino acid for children with inborn errors of ureagenesis (Brusilow 1984), and still has an established role in management of UCDs (Haberle et al 2012).
The role of creatine in ASA is worth considering for several reasons. On one hand, creatine was shown to exhibit neuroprotective potential in vitro (Braissant et al 2002; Bachmann et al 2004; Braissant 2010). As well, ammonium altered creatine biosynthesis and transport in cultured rat brain cells, resulting in a secondary creatine deficiency (Braissant et al 2008). The situation in human patients with ASA is however still unclear. There is some evidence of the importance of arginine for creatine biosynthesis based on low levels of urinary guanidinoacetate (GAA) (Arias et al 2004), an intermediary metabolite of creatine biosynthesis, but further studies in treatment-naïve (before start of arginine supplementation) ASA patients are needed. Such studies should also include the situation in brain since cerebral creatine deficiency would be very relevant for those patients, but not easy to treat since already increased cerebral GAA was demonstrated in a previously reported ASA patient under treatment (Sijens et al 2006).
Agmatine or polyamines deficiencies have not yet been studied in UCDs patients, but remain a theoretical possibility in ASA.

Argininosuccinate and guanidinosuccinic acid toxicity

Both argininosuccinate and guanidinosuccinic acid (GSA) are very likely involved in the pathophysiology of ASA, although this is mainly based on assumption and less on experimental evidence. The best arguments supporting argininosuccinate toxicity are from a randomized, double-blind, placebo-controlled, cross-over study in ASA patients, in whom

administering higher doses of arginine resulted in increases in transaminases (Nagamani et al 2012). This study and the high prevalence of chronic liver disease in ASA supported that argininosuccinate likely contributes to this liver toxicity (Bigot et al 2017), which is in this pattern not seen in other UCDs, apart from arginase deficiency.

GSA arises from the oxidation of argininosuccinate in the presence of free hydroxyl and superoxide radicals (Aoyagi et al 1996; Aoyagi et al 1996). GSA is not only a uremic toxin increased in states of nitrogen retention, such as renal failure, but in addition, together with other guanidino compounds accused to contribute to the (neuro)toxicity in ASA. Interestingly, but not yet studied in human UCD patients, concentrations of GSA in both serum and urine decline sharply in animals and humans exposed to methionine (Cohen 2003) and this could be a therapeutic alternative.

NO deficiency

In addition to its catalytic requirement for arginine synthesis, ASL has been shown to be structurally essential for the formation of the NO-synthesis-complex that generates NO. For this reason, ASA patients are considered NO deficient and at risk for high blood pressure (Fakler et al 1995; Brunetti-Pierri et al 2009; Nagamani et al 2012). Indeed, individuals with ASA have anecdotally been reported to suffer from elevated blood pressure as compared to the normal values in the general population (Brunetti-Pierri et al 2009). To further understand the role of ASL in blood pressure regulation, a hypomorphic mouse model for ASA was generated ($Asl^{Neo/Neo}$). These mice have less than 20% residual ASL activity, recapitulating the human ASA patients' phenotype. Biochemically, $Asl^{Neo/Neo}$ mice have plasma amino acid profiles with elevation of citrulline and argininosuccinate, and low arginine. Clinically, $Asl^{Neo/Neo}$ survive three weeks during which they suffer from a significant growth restriction, a multi-organ dysfunction characterized by elevation of liver transaminases, decreased renal creatinine clearance and high systolic and diastolic blood pressure (Erez et al 2011).

ASL role in blood pressure regulation

Mechanistically, $Asl^{Neo/Neo}$ mice demonstrate a reduction in NO levels resulting in reduced NO-mediated vascular vasodilatation following acetylcholine-induced relaxation (Erez et al 2011). This impairment in NO synthesis in general and specifically in the endothelial cells of blood vessels causes the hypertension in $Asl^{Neo/Neo}$ mice. In support, liver-targeted ASL gene therapy, did not rescue the high blood pressure in $Asl^{Neo/Neo}$ mice, emphasizing the tissue-specific importance of ASL expression (Nagamani et al 2012). Only NO donor

supplementation was able to restore normal blood pressure levels in the gene therapy treated ASL-deficient mouse (Nagamani et al 2012).

In a subsequent work, Jordan et al (Kho et al 2018) showed that by similar mechanism, knockout of ASL in endothelial cells is sufficient to cause high blood pressure in mice. In this work, the authors showed that mice that do not express ASL in endothelial cells had high systolic and diastolic blood pressures as compared to control mice, when grown with arginine, nitrite and nitrate free diet. Additionally, the authors demonstrate that endothelial cells from ASA patients have lower levels of NO in comparison to endothelial cells of healthy individuals (Kho et al 2018). Interestingly, preliminary results suggest that ASL is also involved in the central regulation of blood pressure, specifically in response to stress (Erez, personal communication).

An ASA patient that suffered from chronic hypertension, did not respond to conventional multi-therapy but normalised his blood pressure with NO donors (Nagamani et al 2012).

Protective role of ASL in the gut

The essentiality of ASL and arginine for NO synthesis is further emphasized in the gut, where NO can either be pro-inflammatory or protective (Alican and Kubes 1996; Shah et al 2004; Blaise et al 2005). In fact, ASL seemed essential to protect newborn mice from premature enterocolitis (Premkumar et al 2014). In subsequent work, knockout of ASL from the different intestinal cells involved in colitis resulted in opposite consequence: while knockout of ASL from gastrointestinal immune cells was beneficial, depletion of ASL from the epithelial intestine was detrimental and increased colitis severity (Stettner et al 2018). Furthermore, depletion of ASL and consequently decreasing NO levels in the intestinal villi, increased the development of inflammation induced colon cancer. For translational implications, the authors further demonstrate that inducing NO-synthesis by supplementing colitis mice with citrulline as substrate for the NO pathway, together with Fisetin which upregulates ASS and ASL levels, increases NO levels more specifically in epithelial cells and hence improves the epithelial cells' integrity and alleviates colitis severity (Stettner et al 2018).

Cerebral oxidative/nitrosative stress

Brain analysis in *Asl*$^{Neo/Neo}$ mice display increased cerebral levels of nitrite and nitrate, downstream metabolites of NO, in favour of oxidative stress and a diffuse neuronal disease revealed by the accumulation of nitrotyrosine, a marker of nitrosative stress. Nitrosative stress

is caused by reactive nitrogen species such as NO and superoxide (O_2^-), which forms peroxynitrite, a highly damaging compound (Baruteau et al 2018).

NOS are dimeric enzymes, which generate NO when 2 monomers are coupled. Low intracellular arginine causes NOS uncoupling in ASA (Erez et al 2011; Nagamani et al 2012), which generates superoxide and peroxynitrite (Lin et al 2003; Pignitter et al 2006) **(Figure 4)**. Peroxynitrite reacts with tyrosine motifs and generates nitrotyrosine, which causes structural modification and protein inactivation (Nakamura and Lipton 2017). Neurons are more prone to oxidative/nitrosative stress and rely on glutathione supply from astrocytes, which can induce gamma-glutamyl transpeptidase to restore their stock of reduced glutathione (Gegg et al 2003).

A gene therapy approach was designed to cure this neurological disease in $Asl^{Neo/Neo}$ mice. A liver-directed gene therapy showed a negligible decrease of cerebral nitrosative/oxidative stress. Conversely, a combined strategy targeting cerebral neurons and hepatocytes displayed a dramatic improvement. This correlated with decrease of cortical cell death and improved behavioural testing (Baruteau et al 2018). This demonstrates that the neurological disease in ASA has a dual origin: i) neurotoxicity caused by hyperammonaemia and ii) a neuronal disease associated with nitrosative/oxidative stress independent of hyperammonaemia. These findings confirm the clinical observation that a therapeutic approach exclusively centred on normalising ammonaemia is ineffective to treat the neurological disease.

Unpublished work shows ASL predominantly expressed in the locus coeruleus (LC), a brainstem region that secretes catecholamines and the main source of norepinephrine in the brain (Erez, personal communication). In this work, ASL is shown to regulate catecholamine synthesis in the LC in an NO dependent manner. Depletion of ASL from the LC, alters catecholamine levels and results in physiological and behavioural abnormalities in response to stress, which resemble the clinical manifestation observed in ASA patients. Importantly, treatment with NO donors benefits the stress response in LC-ASL deleted mice.

5. Therapeutic perspectives and monitoring

Creatine

Since arginine in most UCDs becomes an essential amino acid, it has been proposed to monitor plasma creatine concentrations and dose arginine accordingly to achieve normal

creatine levels (Boenzi et al 2012). Likewise, the recently revised UCD guidelines (https://www.awmf.org/uploads/tx_szleitlinien/027-006l_S3_Diagnostik-Therapie-Harnstoffzyklusstoerungen_2018-06.pdf) recommend to assess creatine "especially in patients with OTC deficiency, ASS deficiency, and HHH syndrome, as in these disorders low creatine concentrations were found along with other changes in creatine metabolism but the practical consequences of a found low creatine are unclear".

Limiting arginine intake

Arginine has been, besides low-protein diet, for many patients the sole therapy for many years. Different from other UCDs, this regimen was sufficient to avoid hyperammonaemic decompensations and to guarantee metabolic stability. However, since the pathogenesis of liver involvement in this condition was unclear, it was speculated that "the accumulation of argininosuccinate upstream of the metabolic block, the deficiency of arginine and its metabolites downstream of the block, or NO deficiency may have a role in causation of hepatic complications" (Nagamani et al 2012). A trial was undertaken clearly demonstrating that higher doses of arginine in ASA patients results in elevations of liver transaminases (Nagamani et al 2012). From this, the recommendation to avoid high-dose arginine but to use, in combination with ammonia scavengers, low-dose arginine instead was made (Haberle et al 2012; Nagamani et al 2012).

Anti-oxidants

In *Asl*$^{Neo/Neo}$ mice, decreased levels of reduced glutathione were observed in liver and partially improved after liver-directed gene therapy (Baruteau et al 2018). Markers of oxidative stress were elevated in murine plasma, urine, and in tissues (Erez et al 2011; Nagamani et al 2012). Taken together, these observations suggest that oxidative stress is a pathophysiological mechanism in ASA. No antioxidant supplementation has been reported in ASA patients.

NO donors

In vivo studies in murine models of ASLD and *in vitro* studies performed using cells derived from individuals with ASA have shown that dysregulation of NO signalling may be an important contributor to the phenotype (Erez et al 2011; Nagamani et al 2012; Baruteau et al 2018; Kho et al 2018). NOS-independent NO supplementation has been shown to improve weight gain and survival, and correct endothelial dysfunction and hypertension in murine models of ASA (Erez et al 2011; Kho et al 2018). These preclinical data suggest that NO

supplementation may present a new therapeutic modality for the treatment of individuals with ASA.

The supplementation of NO in humans is typically accomplished by formulations that contain nitrates and nitrites, which were traditionally considered to be inert compounds generated as end-products of NO metabolism. However, it is now clear that they are recycled in blood and tissues to generate NO (Kapil et al 2014; Bryan and Ivy 2015). The nitrate–nitrite–NO pathway is a major mechanism to by-pass the NOS system to generate NO, which could be particularly relevant in ASA. Nitrates and nitrites have been investigated for the treatment of many cardiovascular conditions including systemic and pulmonary hypertension, peripheral vascular disease, and myocardial infarction (Lundberg et al 2008; Kapil et al 2010; Kapil et al 2014; Omar et al 2016). In an uncontrolled, proof-of-principle study, one ASA individual who had hypertension that was refractory to treatment with multiple antihypertensive medications, was treated successfully with isosorbide dinitrate and subsequently with a custom formulated inorganic nitrite (Nagamani et al 2012). Additionally, in this individual, NO supplementation was associated with an improvement in some neuropsychological parameters pertaining to verbal memory and nonverbal problem solving. Based on these preliminary results, the safety and efficacy of NO supplementation in ASA is being investigated in two double-blind, randomized, placebo-controlled, crossover clinical trials. The first trial (NCT02252770) is assessing the 2 weeks effect of NO supplementation on flow-mediated dilatation of brachial artery, an *in vivo* marker of endothelial function. The second (NCT03064048) is assessing the 24 weeks effect of NO supplementation on neurocognition in the domains of general cognition, memory, executive and fine motor functioning.

Different pathogenic variants in *ASL* are likely to have varying effects on the catalytic versus structural properties of ASL. Thus, some ASA individuals may have significant NO deficiency whereas others may have none. Currently, based on the genotypic information, it is difficult to ascertain as to which patients would benefit from NO supplementation; however, it would be reasonable to assume that individuals with hypertension or abnormal flow-mediated dilatation are more likely to benefit.

Gene therapy

Correction of the ureagenesis has been successfully reported in $Asl^{Neo/Neo}$ mice with adenoviral (Nagamani et al 2012) or adeno-associated viral (AAV) vectors serotype 8 with either a murine (Baruteau et al 2018) or human (Ashley et al 2018) *ASL* transgene. Rescue of survival and normalisation of ammonia levels were observed with improvement of locomotor testing

(Baruteau et al 2018). The long-lasting rescue of the phenotype was documented up to one year after a single systemic injection of gene therapy when the experiment was terminated (Baruteau et al 2018). This liver-targeted approach could benefit ASA patients at high risk of hyperammonaemic decompensation.

However a liver-restricted approach will not fully correct the neurological disease as suspected in liver-transplanted ASA patients (Robberecht et al 2006; Marble et al 2008) and in the *Asl*$^{Neo/Neo}$ mouse (Baruteau et al 2018). A translational approach, which could benefit most ASA patients, would be to consider a gene therapy targeting i) hepatocytes to rescue the urea cycle and ii) cerebral neurons to restore neuronal ASL activity and correct the primary neuronal disease. Further preclinical work needs to be undertaken to know to what extent older patients with severe neurological phenotype could benefit from late brain-directed therapy.

Proposal for monitoring of ASA patients

ASA patients develop a systemic disease overtime. Developmental delay is usually diagnosed in late infancy, epilepsy and liver symptoms in early childhood, arterial hypertension, ataxia and hypokalaemia in late childhood and adolescence (Baruteau et al 2017). Therefore it is critical to monitor carefully these patients **(Table 4)**.

Conclusion

Six decades after its first description, ASA retains various unanswered questions. A complex clinical presentation and the challenge to prevent a neurological phenotype, despite conventional therapy and normal ammonia levels, have generated pathophysiological hypotheses highlighting alternative roles of ASL outside the urea cycle. The generation of ASL-deficient mouse models has enabled a better understanding of the physiological importance of ASL in endothelial cells, enterocytes and brain. Thus this complex disease progressively enables a better understanding of the role of urea cycle-related enzymes outside the liver. Identifying these pathogenic mechanisms is key to optimise therapy. Alternative therapeutics such as NO donors, anti-oxidants, creatine supplementation and/or restoration of *in situ* ASL expression by gene therapy or editing directed to various organs will hopefully add to a better therapy and outcome in this disease.

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