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# Elevated plasma concentrations of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine in citrullinemia

Thomas Lücke[a,*,1], Dimitrios Tsikas[b,1], Nele Kanzelmeyer[a], Bernhard Vaske[c], Anibh Martin Das[a]

[a]*Department of Pediatrics, Medical School of Hannover, Carl Neuberg Str. 1, D-30625 Hannover, Germany*
[b]*Institute of Clinical Pharmacology, Medical School of Hannover, D-30625 Hannover, Germany*
[c]*Biometric Institute, Medical School of Hannover, D-30625 Hannover, Germany*

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**Abstract**

Citrullinemia is an inborn error of the urea cycle with deficiency of the argininosuccinate synthase. It is characterized by elevated concentrations of L-citrulline and decreased levels of L-arginine in body fluids. Asymmetric dimethylarginine is an endogenous inhibitor of nitric oxide synthase that converts L-arginine to L-citrulline and nitric oxide (NO). Asymmetric dimethylarginine is hydrolyzed by the enzyme dimethylarginine dimethylaminohydrolase to L-citrulline and dimethylamine. Elevation of L-citrulline in citrullinemia prompted us to study the L-arginine/NO pathway in this disorder. In 8 children with citrullinemia (3 days to 3 years of age), elevated plasma levels of asymmetric dimethylarginine ($P = .028$) were found compared with age-matched healthy children. We hypothesize that the L-arginine/NO pathway plays a role in the pathophysiology of citrullinemia.


## 1. Introduction

Citrullinemia (MIM 215700) is an inborn error of the urea cycle (autosomal recessive trait, chromosome 9q34) due to argininosuccinate synthase deficiency and results in dramatically elevated levels of L-citrulline, especially during catabolic spells. Citrullinemia is often associated with severe mental retardation after metabolic decompensation [1]. The mechanisms leading to cerebral damage in citrullinemia are incompletely understood. Ischemia, energy depletion, ammonia-associated neurotoxicity, and enhanced oxidative stress during metabolic decompensation are currently discussed as potential factors contributing to the often poor neurologic outcome in citrullinemia [2]. A role of L-citrulline itself in the pathogenesis of citrullinemia is uncertain.

By far the main source of L-citrulline in the body of patients with citrullinemia is the urea cycle. However, L-citrulline is also produced in the L-arginine/nitric oxide (NO) pathway [3]. The NO synthases (NOSs) are a family of enzymes that convert L-arginine to L-citrulline and NO [4]. The activity of NOS is effectively controlled by endogenous inhibitors, with the L-arginine analogue asymmetric dimethylarginine (ADMA) being the most important [5,6]. Asymmetric dimethylarginine is produced by methylation of protein-associated L-arginine via *N*-methyl protein transferases [7]. After proteolysis, ADMA is released into the circulation from which it is eliminated in part by the kidney [7,8].

Another, much more abundant contributor to L-citrulline in the L-arginine/NO pathway involves the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes ADMA to L-citrulline and dimethylamine (DMA) [7]. Rough estimates indicate that approximately 70% of daily ADMA production is eliminated by the kidney as DMA [9]. In brain tissue, ADMA and DDAH are abundantly present [7].

Nitric oxide is a gaseous, freely diffusible molecule with multiple physiologic functions including vasodilation, inhibition of platelet aggregation, and adhesion, as well as neurotransmission [10]. Prompted by its close connection to L-citrulline [3], we investigated in the present study the status of the L-arginine/NO pathway in citrullinemia. Nitric oxide synthesis was assessed by measuring nitrite and nitrate, the

* Corresponding author. Tel.: +49 0511 532 3220; fax: +49 0511 532 8073.
*E-mail address:* luecke.thomas@mh-hannover.de (T. Lücke).
[1] These authors contributed equally to the study.

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major NO metabolites and indicators of NO synthesis in plasma and urine [11]. The synthesis, metabolism, and elimination of ADMA were assessed by measuring circulating and excretory ADMA, and urinary DMA, which served as a rough estimate of whole-body DDAH activity [9].

If the L-arginine/NO pathway played a role in the pathophysiology of citrullinemia, alterations of its parameters should be expected in citrullinemic patients.

## 2. Methods

Eight anabolic patients with citrullinemia and 8 age-matched healthy controls were investigated. The study was approved by the ethics committee of the Hannover Medical School (Hannover, Germany), and written informed consent was obtained from the parents.

Asymmetric dimethylarginine in plasma and urine was determined by gas chromatography-tandem mass spectrometry as described elsewhere [12].

Nitrate and nitrite in plasma and urine were determined simultaneously by gas chromatography-mass spectrometry as described previously [13].

Urinary creatinine was determined by high-performance liquid chromatography as described recently [14].

Dimethylamine in urine was determined by gas chromatography-mass spectrometry as described elsewhere [15].

Sample size for the single parameters varied because of the limited amount of urine and blood available in young children.

L-Arginine, L-citrulline, and glutamine were determined using a commercial amino acid analyzer.

Data from patients and healthy controls were compared using the Wilcoxon test (SPSS, version 13: SPSS, Chicago, IL). Data are presented as mean ± SD. Values of $P < .05$ were considered significant.

## 3. Results

The plasma amino acid profiles and the ammonia levels of the patients with citrullinemia at the time of our study are summarized in Table 1.

Plasma levels of ADMA in the patients with citrullinemia were significantly higher than in controls (1253 ± 405 vs 856 ± 159 nmol/L, $P = .028$), but there was some overlapping (Fig. 1A). Renal excretion of ADMA was slightly but not significantly higher in patients with citrullinemia (26.1 ± 30 vs 12.3 ± 9 μmol/mmol creatinine, $P = .068$) (Fig. 2A).

In plasma, neither nitrate (73.5 ± 15.3 vs 66.5 ± 31.1 μmol/L, $P = .345$) (Fig. 1B) nor nitrite (2.98 ± 1.5 vs 2.8 ± 1.4 μmol/L, $P = .5$) (Fig. 1C) differed in both groups. In addition, urinary creatinine-corrected excretion of nitrate (258 ± 198 vs 267 ± 305 μmol/mmol creatinine, $P = 1.0$; Fig. 2B) and nitrite (1.6 ± 1.7 vs 0.36 ± 0.32 μmol/mmol creatinine, $P = .109$; Fig. 2C) did not differ in both groups.

Table 1
Ammonia and amino acid plasma levels in 8 anabolic patients (3 days [n = 1], 3 weeks [n = 1], 1 year [n = 1], 2 years [n = 4], 3 years [n = 1] of age) with citrullinemia investigated in the present study

| Parameter | Mean | Range (μmol/L) | Reference range |
|---|---|---|---|
| Arginine | 81 | 22-170 | 36-139 |
| Citrulline | 1059 | 299-2092 | 3-42 |
| Glutamine | 381 | 50-759 | 279-794 |
| Ammonia | 56 | 22-129 | <50 |

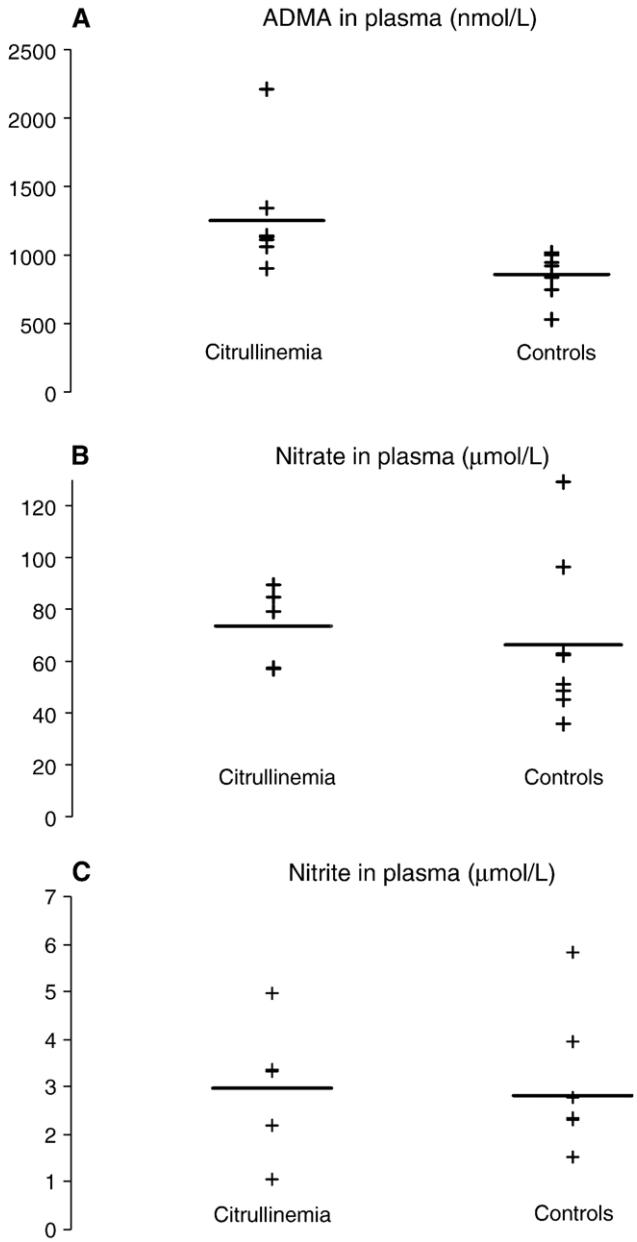


Fig. 1. Plasma levels of ADMA (A), nitrate (B) and nitrite (C) in children with citrullinemia ("Citrullinemia," n), and in healthy children ("Controls," m). Horizontal bars indicate the mean values. Asymmetric dimethylarginine levels were significantly higher in Citrullinemia as compared with Controls ($P = .028$; n = 8, m = 8). No statistically significant differences were found between Citrullinemia and Controls for nitrate ($P = .345$; n = 5, m = 8) and nitrite ($P = .5$; n = 5, m = 8).

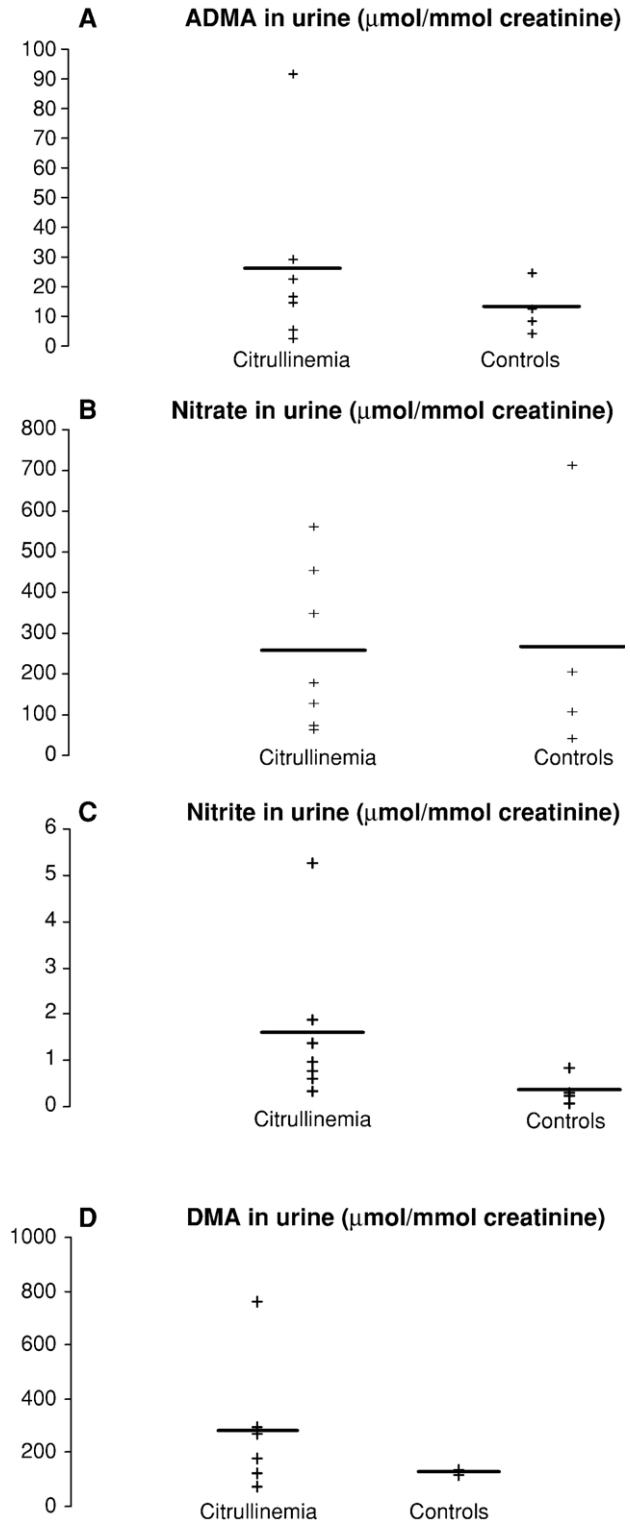


Fig. 2. Creatinine-corrected urinary levels of ADMA (A), nitrate (B), nitrite (C), and DMA (D) in children with citrullinemia ("Citrullinemia," n) and in healthy children ("Controls," m). Horizontal bars indicate the mean values. No statistically significant differences were found between Citrullinemia and Controls for ADMA ( $P = .068$; n = 8, m = 4), nitrate ( $P = 1.0$; n = 7, m = 4), nitrite ( $P = .36$; n = 7, m = 4), and DMA ( $P = 0.144$; n = 6, m = 4).

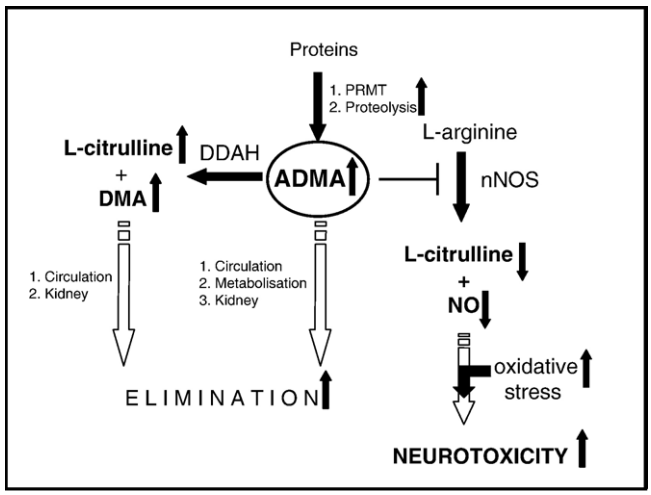


Fig. 3. Proposal of the L-arginine/NO pathway in neuronal cells in citrullinemia. Elevated activities of protein arginine methyltransferase and of proteolytic enzymes lead to elevated ADMA levels, which markedly inhibit nNOS activity. Suppressed nNOS activity and elevated oxidative stress remarkably reduce NO production and bioavailability and concomitantly increase nitrative stress within the neuron leading both to NO-related dysfunction (including ischemia and cytotoxicity) and neurotoxicity. The enzyme DDAH converts ADMA to L-citrulline and DMA. L-Citrulline, ADMA, and DMA from the cerebrum and other organs reach the circulation from which they are eliminated by the kidneys. On the assumption that urinary DMA exclusively comes from ADMA, it is estimated that approximately 90% of daily produced ADMA are eliminated via DDAH, whereas only 10% are excreted in unchanged form via urine in citrullinemia. Superscript and subscript arrows indicate elevated and decreased concentration or biological activity, respectively.

The urinary excretion rate of DMA was not significantly higher in patients with citrullinemia as compared with healthy controls (285 ± 249 vs 127 ± 9.9 μmol/mmol creatinine, $P = .144$; Fig. 2D).

No correlation was found between ADMA in plasma and ADMA in urine, ADMA in plasma and DMA in urine, ADMA in urine and DMA in urine, L-citrulline and ADMA in plasma, L-citrulline and ADMA in urine, or L-citrulline and DMA in urine (data not shown).

## 4. Discussion

Elevated circulating levels of ADMA have been found in a variety of diseases, most of them being associated with NO-dependent endothelial dysfunction [16,17]. Asymmetric dimethylarginine is considered to be a novel marker for cardiovascular disease [18]. To our knowledge, the present study reports for the first time elevated ADMA levels in citrullinemia.

Urinary excretion rates of ADMA in citrullinemia and controls did not differ significantly, suggesting that the elevation of ADMA levels in the circulation is not a result of impaired renal elimination. To our present knowledge, DDAH-catalyzed hydrolysis of ADMA to L-citrulline and DMA represents the major degradative metabolic pathway of ADMA [9]. Dimethylamine excretion rate seems to be higher in

citrullinemia as compared with controls (though not significantly), suggesting that ADMA degradation to DMA and L-citrulline by DDAH is increased in this disease. Asymmetric dimethylarginine elevation in citrullinemia is therefore probably due to increased synthesis of ADMA caused either by an activation of the protein-arginine methyltransferase I or by an increased availability of its substrate (Fig. 3).

Citrullinemia is due to an inborn error in the hepatic urea cycle that leads to a dramatic increase in L-citrulline levels both in blood and cerebrospinal fluid [19]. Asymmetric dimethylarginine hydrolysis via the DDAH pathway is an additional source of L-citrulline [7]. As DMA is increased in some patients, this process may contribute to elevated L-citrulline levels in citrullinemia. It has previously been shown that DDAH is abundantly expressed in the brain, and ADMA concentrations are high in the brain (1-2 $\mu$mol/g protein) [20]. Therefore, there might be considerable local production of L-citrulline in the brain of citrullinemic children via the DDAH pathway. Elevated citrulline levels could lead to reduced intracellular availability of L-arginine and/or feedback inhibition of NOS (Fig. 3); however, further experimentation is needed to prove this hypothesis.

Asymmetric dimethylarginine inhibits all known NOS isoforms. Based on nitrite and nitrate concentration in plasma and urine [11], the results of the present study suggest that whole-body NO synthesis is not altered in citrullinemia, despite higher plasma levels of ADMA as compared with healthy children. However, unaltered urinary excretion of NO metabolites in citrullinemia does not exclude the possibility of a local inhibition of NO synthesis by ADMA, especially in the brain (Fig. 3). It may be speculated that the increase in ADMA levels is more pronounced in brain than in other organs. Interestingly, ADMA is a potent ($IC_{50}$, 1.5 $\mu$mol/L), noncompetitive inhibitor ($K_i$, 0.4 $\mu$mol/L; $K_{ii}$,1.6 $\mu$mol/L) of the neuronal isoform of NOS (nNOS) [6]. However, the endothelial isoform of NOS is only weakly inhibited by ADMA ($IC_{50}$, 12 $\mu$mol/L) in a competitive manner ($K_i$, 3.9 $\mu$mol/L) [6]. Therefore, at saturating levels of L-arginine ($K_M$, 2-7 $\mu$mol/L), as observed in the citrullinemic children investigated in the present study (Table 1), a moderate increase in the ADMA levels within the cells, equivalent to the increase from 902 to 2209 nmol/L in the circulation observed in our study, would not markedly affect the endothelial isoform of NOS activity, for example, in the cardiovascular system. However, increased ADMA levels in the brain of this order of magnitude would considerably inhibit NO synthesis in the neurons (Fig. 3).

Local inhibition of NO synthesis in neuronal cells by ADMA would significantly decrease NO bioavailability in the cerebrum and cause NO-related dysfunctions including ischemia, oxidative stress, and cytotoxicity (Fig. 3). As NO interacts with mitochondrial respiratory chain complexes a lack of NO could lead to altered redox potentials within the respiratory chain resulting in oxidative stress [21].

At present, there is sufficient evidence to place oxidative and nitrative processes in the center of the pathogenic mechanisms that lead to neurotoxicity and neuronal loss, in diseases such as the Parkinson syndrome and amyotrophic lateral sclerosis [22]. In human and animal studies the contribution of NO to neuronal injury by both the nNOS and the inducible form of NOS is well documented [22]. Interestingly, nitration has also been associated with compromised integrity of the blood-brain barrier in multiple sclerosis [22]. Oxidation and nitration of proteins, DNA, and lipids are markers of neurotoxicity in postmortem tissues, particularly from patients with Parkinson and Alzheimer diseases.

The pathophysiology of neurologic damage in citrullinemia is unclear. Hyperammonemia seems not to be the only cause for cerebral damage [23]. The severity of neuropathologic changes seems to correlate with L-citrulline concentration in blood and cerebrospinal fluid [19], but it is unclear whether L-citrulline at high doses is itself a toxic agent [1]. Increased levels of the potent nNOS inhibitor ADMA in citrullinemia may lead to disturbed NO metabolism and/or enhanced nitrative stress in neurons that eventually cause neurologic dysfunction and damage (Fig. 3). It is possible that elevated ADMA synthesis and increased DDAH activity contribute to high cerebral L-citrulline concentrations. Nevertheless, NO-mediated neurotoxicity in citrullinemia remains elusive. Further studies in animal models and in humans are needed. A better understanding of the role of the complex L-arginine/NO pathway in citrullinemia could lead to promising novel therapeutic interventions modifying NO-related dysfunction and NO-dependent oxidative stress in this disease.

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