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# Minimal Ureagenesis is Necessary for Survival in the Murine Model of Hyperargininemia Treated by AAV-based Gene Therapy

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## Abstract

Hyperammonemia is less severe in arginase 1 deficiency compared with other urea cycle defects. Affected patients manifest hyperargininemia and infrequent episodes of hyperammonemia. Patients typically suffer from neurological impairment with cortical and pyramidal tract deterioration, spasticity, loss of ambulation, seizures, and intellectual disability; death is less common than with other urea cycle disorders. In a mouse model of arginase I deficiency, the onset of symptoms begins with weight loss and gait instability which progresses to development of tail tremor with seizure-like activity; death typically occurs at about two weeks of life. Adeno-associated viral vector gene replacement strategies result in long-term survival of mice with this disorder. With neonatal administration of vector, the viral copy number in the liver greatly declines with hepatocyte proliferation in the first 5 weeks of life. While the animals do survive, it is not known from a functional standpoint how well the urea cycle is functioning in the adult



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animals that receive adeno-associated virus. In these studies we administered [1-$^{13}$C] acetate to both littermate controls and adeno-associated virus-treated arginase 1 knockout animals and examined flux through the urea cycle. Circulating ammonia levels were mildly elevated in treated animals. Arginine and glutamine also had perturbations. Assessment thirty minutes after acetate administration demonstrated that ureagenesis was present in the treated knockout liver at levels as low at 3.3% of control animals. These studies demonstrate that only minimal levels of hepatic arginase activity are necessary for survival and ureagenesis in arginase deficient mice and that this level of activity results in control of circulating ammonia. These results may have implications for potential therapy in humans with arginase deficiency.

**Keywords**

Arginase; Hyperargininemia; AAV; Hepatocyte; Urea Cycle Disorder

## Introduction

Urea is the major end-product of nitrogen metabolism in humans and terrestrial animals. The biochemical pathway required for its synthesis consists of 6 enzymes and 2 membrane transporters[1]. Deficiencies in each of these proteins, or the urea cycle defects, have been identified. Each genetic defect gives rise to a discrete clinical and biochemical phenotype[1–2].

Arginase deficiency is an autosomal recessive distal urea cycle disorder resulting from a loss of arginase 1 (ARG1) activity. ARG1 deficiency typically presents in late infancy to the second year with a syndrome of microcephaly, spasticity, seizures, clonus, loss of ambulation, and failure to thrive associated with hyperargininemia[3]. Profound hyperammonemia is uncommon. Neonatal presentations are rare and often are associated with severe hyperammonemia[4–5]. Long-term therapy for arginase deficiency involves a low-protein diet and administration of sodium benzoate and sodium or glycerol phenylbutyrate. These dietary and pharmaceutical interventions can partially alleviate hyperargininemia; however no completely effective therapy is available.

We have developed a gene therapy approach for ARG1$^{-/-}$ animals. Our approach uses an adeno-associated viral (AAV) vector expressing murine *ARG1* administered intravenously on the second day of life[6–8]. We utilized AAV driven by the ubiquitously expressing chicken β-actin promoter/CMV enhancer (CBA) or the liver-specific promoter thyroxine binding globulin (TBG) resulting in hepatic expression of arginase 1. However, we observed a substantial loss of AAV vector genomes and consequent low levels of hepatic arginase activity because of frequent hepatocyte cell division during early life[6, 9–10]. Arginase activity can be detected by a colorimetric enzymatic assay resulting in the production of urea; however when the level of arginase expression is low, exact activity is difficult to quantify by this method.

The measurement of urea production via isotopic methods using metabolic tracers has had an important role in studying urea cycle flux in patients with defects in the urea cycle[11]. Such studies could also be employed to evaluate the efficacy of therapeutic interventions, in this case AAV-based hepatic gene transfer in the treatment of arginase deficiency in a

murine model. This would allow us to assess residual ureagenesis and define the minimal activity necessary for control of plasma ammonia, survival, longevity, and normal cognitive development[7] in these animals. The kinetic approach also avoids limitations of the conventional enzyme assay which result from extremely low arginase activity. Furthermore knowing the amount of activity that is necessary for survival and urea cycle functioning in arginase-deficient animals may be important in planning future human interventions.

In these studies, we used mass spectrometry to monitor the *in vivo* synthesis of [$^{13}$C] urea following administration of [1-$^{13}$C] acetate. Hepatic mitochondria, the location of the first steps of ureagenesis, quickly convert labeled [1-$^{13}$C] acetate to $H^{13}CO_3^-$ in the tricarboxylic acid cycle. Some of the metabolite then becomes substrate for carbamoyl phosphate synthetase yielding [$^{13}$C] carbamoyl phosphate which the urea cycle converts to [$^{13}$C] urea[11]. The remainder of the $H^{13}CO_3^-$ appears as $^{13}CO_2$ in blood and in exhaled air. By measuring [$^{13}$C] urea in blood of the mice, determination of the urea pool turnover is possible[11]. This method of investigation has provided us with an assessment of how well the urea cycle is functioning in stable adult ARG1$^{-/-}$ animals treated by AAV-based liver-specific gene therapy. The purpose of this report is to present the outcomes and potential implications of this study.

## Results

### Animal Survival

Survival of animals undergoing interventions versus no therapy was performed. We observed no difference in survival of AAV-treated ARG1$^{-/-}$ mice vs. littermate controls at the time of study ($p=0.43$). Animals were euthanized at the completion of these studies. As expected, all untreated knockout animals perished before weaning (Figure 1) by about two weeks of life ($p<0.0001$ when compared with AAV-treated ARG1$^{-/-}$ mice or controls).

### Liver Examination

We previously noted that AAV-treated animals were lean compared to their littermate controls[6]. We therefore compared liver weights between the groups and we found that this parameter, as a proportion of body weight, differed between groups with treated ARG1$^{-/-}$ livers being about 27% heavier more than that of littermate controls. AAV-treated ARG1$^{-/-}$ mice had livers that weighed 5.45 ± 0.53 grams (n=10) compared to that of littermate controls with 4.30 ± 0.30 grams (n=10) ($p=0.00001$). In addition, gross examination of liver showed a tan color in the AAV-treated ARG1$^{-/-}$ and a reddish brown color in controls (Figure 2A, B). The organs were soft without evidence of cirrhosis and were indistinguishable from controls when examined by palpation alone.

### Plasma Ammonia and Biochemical Analysis

To determine in part how well the urea cycle was functioning, plasma ammonia concentration was measured before administration of [1-$^{13}$C] acetate at time 0. Mice were studied at 10–12 weeks of age. Baseline plasma ammonia concentration did differ between the two groups with AAV-treated ARG1$^{-/-}$ mice having circulating ammonia levels about 85% higher than that of littermate controls. Littermate controls (n=10) had plasma ammonia

concentrations on average of 104.03 ± 46.27 μmol/L while plasma ammonia concentrations in AAV-treated knockout mice (n=9) were 192.35 ± 82.12 μmol/L (p=0.01) (Figure 3).

We also noted changes in amino acids levels before and after acetate administration (Table 1). AAV-treated ARG1$^{-/-}$ mice showed higher levels of plasma glutamine at baseline, but both littermate controls (n=11) and AAV-treated ARG1$^{-/-}$ animals (n=11) responded to acetate injection with a comparable (>20%) decline in the plasma glutamine concentration. Similarly littermate controls (n=11), showed a decline (>20%) of blood arginine; in contrast, acetate treatment evoked a slight increase (~3%) of blood arginine in AAV-treated ARG1$^{-/-}$ mice (n=11). Similarly citrulline declined in the littermate controls (n=11) and increased in AAV-treated mice (n=11). The AAV-treated animals (n=6) demonstrated hypoornithinemia compared to the littermates (n=11); circulating plasma ornithine in the AAV-treated ARG1$^{-/-}$ mice at baseline was only 57% of littermate controls. Plasma ornithine in the littermate controls demonstrated a decline of ~17% thirty minutes after acetate administration; however ornithine only declined ~4% in the AAV-treated mice after acetate administration.

### Hepatocellular Arginase Activity and Ureagenesis

Our prior investigations[6, 9] indicated that hepatic arginase expression declined with animal growth as AAV episomes were lost consequent to hepatocellular division. We examined hepatic arginase activity in littermate controls and AAV-treated ARG1$^{-/-}$ mice in these studies. Immunohistochemistry demonstrated scattered arginase-positive cells throughout the liver parenchyma (Figure 2 C, D) compared to littermate controls; no collagen deposition consistent with fibrosis was present. Arginase expression was widespread in the latter animals. Ureagenesis was examined in AAV-treated ARG1$^{-/-}$ mice (n=11) and compared to littermate controls (n=11) by [1-$^{13}$C] acetate administration. The findings demonstrated that the rate of ureagenesis is about 3.3% of control animals (Figure 4).

## Discussion

Urea is the major end-product of mammalian nitrogen metabolism. The arginase 1 single point mutation in our mouse model results in no residual enzyme activity in the liver. We showed previously that arginase 1 knockout mice were able to survive and have normal learning and cognitive function as adults with reduced hepatic enzyme activity after neonatal administration of an AAV vector expressing arginase 1[6–8]. However in the studies conducted herein, we have demonstrated that the livers of the AAV-treated ARG1$^{-/-}$ mice weigh more than the littermate controls. Our group has previously demonstrated that hepatocytes from the ARG1$^{-/-}$ mice are two- to threefold larger than that of controls[12] suggesting that hepatocytes are undergoing hypertrophy and this may be why the organ weights from these animals were greater. The hepatocytes also have been demonstrated to contain a variety of intracytoplasmic inclusions, including large, well-defined, round or oval eosinophilic inclusions[12].

A key question is how much hepatic arginase activity is needed to normalize ureagenesis and allow survival of knockout mice. We addressed this problem by utilizing the stable isotope method to measure *in vivo* flux through the urea cycle. Our extensive experience

with this approach has demonstrated its utility in human investigations[11, 13–17]. It is important to understand the relationship between flux and enzyme activity in order to apply gene transfer technology to human trials and has been employed in these studies to answer this question.

Elevated plasma glutamine, a finding common in most urea cycle disorders, also is increased in the AAV-treated ARG1$^{-/-}$ mice. This abnormality reflects the tendency to hyperammonemia in animals with a limited number of arginase expressing cells in the liver and elsewhere[6, 8]. These animals also are more vulnerable to administration of ammonium chloride, as previously demonstrated[7]. The administration of acetate to these animals and littermate controls caused a decline (~20%) of blood glutamine concentration, perhaps denoting an increase of N-acetylglutamate (synthesized from acetyl CoA and L-glutamate), an obligatory effector of carbamoyl phosphate synthetase 1, the initial step of the urea cycle. The lowered blood glutamine also could reflect a sudden release of insulin[19].

Hepatic mitochondria convert $CH_3{}^{13}COO^-$ to $H^{13}CO_3^-$ in the tricarboxylic acid cycle and then condense the latter with $NH_4$ to carbamoyl phosphate, which mitochondria combine with ornithine to yield citrulline. In the littermate controls, we detect a decline of circulating ornithine of about 18% as it is consumed in the generation of citrulline. Progressing through the urea cycle, plasma citrulline and arginine concentrations also decline (here about 22% from baseline for both) as arginase acts on arginine to generate [$^{13}$C] urea and also regenerate ornithine.

However, in the AAV-treated ARG1$^{-/-}$ mice a different biochemical picture emerges. Plasma arginine and citrulline levels are high because limited hepatic arginase enzymatic activity restricts regeneration of ornithine from arginine. Indeed, baseline ornithine is only about 50% of the value in controls. While plasma ornithine does decline with the administration of acetate as in the littermate controls, the magnitude is substantially less: only about 4% from baseline. Conversely and unlike the littermate controls, plasma arginine and citrulline concentrations *increase* slightly as, we speculate, that there is more flux into the proximal urea cycle due to acetate administration, likely due to the limited hepatic arginase activity. Thus the limited amount of arginase activity allows animal survival[6], control of circulating ammonia, and development of a normal central nervous system[7]. However, the metabolic defect still is associated with increased in hepatic cell mass as seen in the AAV-treated ARG1$^{-/-}$ mice.

While the findings described may have important implications for arginase deficiency, they are not broadly applicable to other disorders of the urea cycle, particularly the proximal disorders. Studies conducted with the ornithine transcarbamoylase deficient (spf)[20–21] and arginosuccinate synthetase deficient[22] murine models demonstrate that the residual expression of enzyme in hepatocytes after AAV administration in the neonatal period are insufficient to prevent hyperammonemia and death. However, in the studies conducted herein the measurement of ureagenesis in these AAV-treated ARG1$^{-/-}$ mice has now allowed for an improved understanding of the relationship between the residual arginase activity and *in vivo* metabolic competency following a single dose of neonatal AAV expressing arginase 1. The findings from these studies have several important implications

and raise further questions. First with the loss of large numbers of episomal AAV vector DNA[6, 8–9], a small amount remains that serves to provide minimal ureagenesis compared with controls. Our prior studies of a conditional knockout of arginase 1 supported this concept and demonstrated that arginase activity of as little as 9% of controls appeared to be necessary for survival in that adult-onset model[23]. The current studies suggest that <5% of control activity leads to normal development in neonatal onset arginase deficiency; however, the urea cycle still is not functioning normally and the animals do remain vulnerable to a nitrogen load[7] but this is sufficient to provide long-term survival.

Second, plasma ornithine concentration remains low in the AAV-treated ARG1$^{-/-}$ mice. The question arises as to whether the administration of ornithine to AAV-treated ARG1$^{-/-}$ mice will improve function by increasing nitrogen flux into the urea cycle. Studies will commence to address this question; however we postulate that this treatment may improve proximal urea cycle function, thus leading to lower circulating plasma ammonia. However, this will not address the limited arginase capacity provided by the relatively small number of arginase-expressing hepatocytes. Furthermore, the urea cycle takes place in the periportal hepatocytes and expression of arginase in hepatocytes by AAV-based expression is heterogenous in its location.

A final question is whether an improvement in urea cycle function will occur with a second administration of AAV expressing arginase 1. Our previous studies have demonstrated that operational tolerance is established to transgene-encoded proteins with neonatal administration of AAV-expressing vectors in mice[6, 10]. Immunologic ignorance appears to be an important mechanism for lack of an immune response to AAV when administered in the neonatal period to mice (unpublished data); these findings and that of others[22] suggest that these animals would benefit from readministration of AAV after adult organ size has been reached, typically by about 5–6 weeks of life[9]. As an associated decline in episomal copy number would be expected in the human liver (albeit over a longer period than the murine liver where the rate of doubling is much more rapid); such questions will be important to address as therapies for arginase deficiency and other metabolic disorders with AAV-based therapy are considered for clinical application.

## Materials and Methods

### Materials

Sodium [1-13C] acetate ([98.0 atom% excess) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Reagents for derivatization of urea and amino acids were from Pierce Chemical Co. (Rockford, IL, USA).

### Mouse Procedures

The targeted ARG1 allele contains a deletion in exon 4 of the ARG1 gene and were generated[12] and backcrossed to achieve a homogeneous NIH-Swiss strain background as described previously[6]. All mice were housed under specific pathogen-free conditions with food and water provided ad libitum. Mice were kept according to the National Institutes of Health guidelines and all experimental procedures were conducted in accordance with

guidelines for the care and use of research animals at our institution. Arginase-deficient newborn pups on the second day of life were injected with $1.0\times10^{14}$ gc/kg of AAVrh10 TBG-mArg1 diluted in pharmaceutical grade saline by the superficial facial vein. The injections were performed in a total volume of 50 μl. 10–12-week-old mice were studied. Mice were fed standard mouse chow (Labdiet/PMI Nutrition International, St. Louis, MO., [Picolab Rodent diet 20, Catalogue Number 5053]). At 8 am of the day of study, chow was removed. At 11 am baseline blood was collected followed by intraperitoneal administration of 1% [1-$^{13}$C] acetate (5.4 μl/gram weight) prepared in sterile pharmaceutical grade saline in a total volume of 500 μl. Thirty minutes later blood was collected and the animals were euthanized. Plasma and serum was frozen immediately and stored at −80°C until analysis. Whole livers were removed and weighed.

### PCR genotyping

Genomic DNA was prepared from tail tip by standard methods and genotyping by PCR was performed as previously described[6].

### Biochemical Analysis of Serum

The concentration of amino acids was determined by HPLC utilizing pre-column derivatization with *o*-phthalaldehyde as previously described[24].

### Ammonia Analysis of Serum

Ammonia was determined in serum samples by reductive amination of 2-oxoglutarate and oxidation of NADPH employing a commercial kit (Sigma-Aldrich, St. Louis, MO) using 10 μl for each sample tested. Results are presented as mean ± SD.

### Immunohistochemistry for Arginase Expression

Livers were removed from euthanized animals and placed in 4% paraformaldehyde for 18–24 hours. Immunohistochemistry was performed as previously described[6].

### Sodium 1-13C Acetate Loading Study

The studies were performed in the morning after food was removed. Baseline samples of blood were collected as well as 30 minute samples. Specimens were centrifuged at 4° to isolate plasma. The measurement of [13C] urea was performed as previously described[16] with appropriate adjustment of volumes based on quantity of mouse plasma.

### Statistical Analysis

Survival curves were computed in each group of mice using the Kaplan-Meier (KM) method and compared across groups using the log rank test. Calculations were made using software JMP PRO version 11 (SAS, Cary, NC). T test was utilized for other statistical analyses for other comparisons. P values of $<0.05$ were considered significant.

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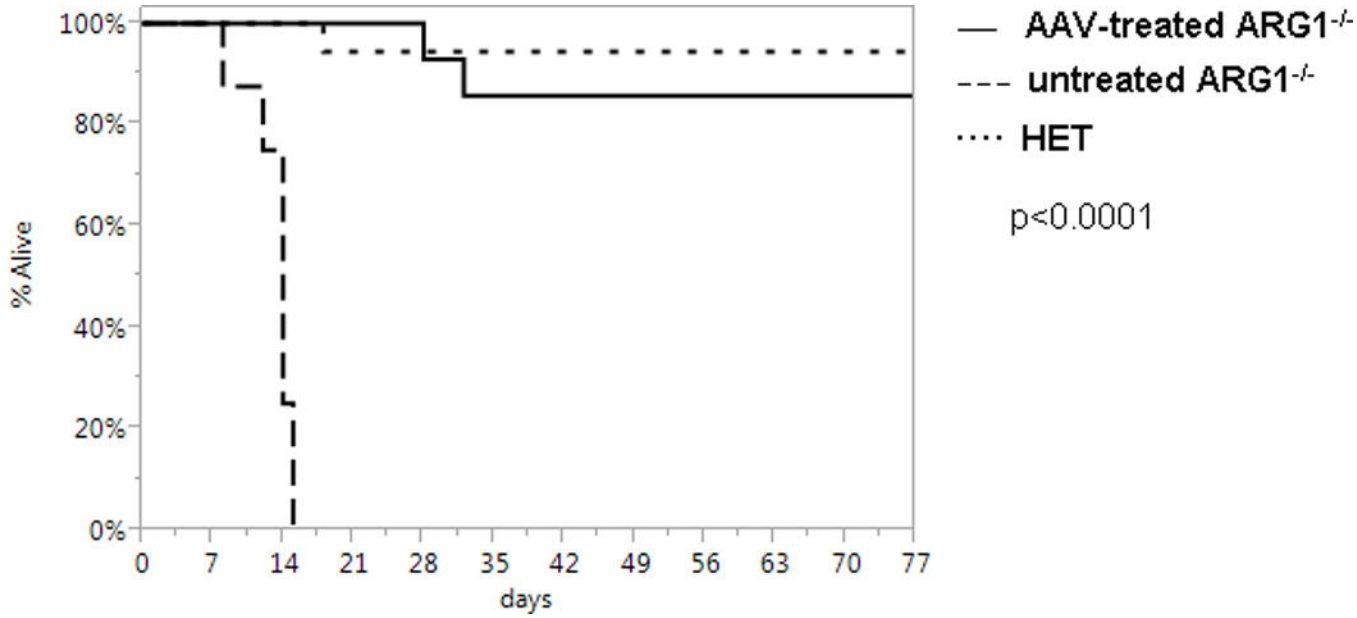


**Figure 1. Survival of *ARG1*$^{-/-}$ mice**

Survival in days of untreated ARG1$^{-/-}$ mice (n=8), intravenous-injected rAAVrh10-co-Arginase *ARG1*$^{-/-}$ mice (n=14), and untreated littermate controls (n=18). The *ARG1*$^{-/-}$ mice treated with AAVrh10-co-Arginase exhibited a marked improvement in survival when compared to that of the untreated *ARG1*$^{-/-}$ mice. (p=0.43 for treated compared with littermate controls and p<0.0001 when AAV-treated ARG1$^{-/-}$ mice or littermates were compared to untreated *ARG1*$^{-/-}$ mice.)

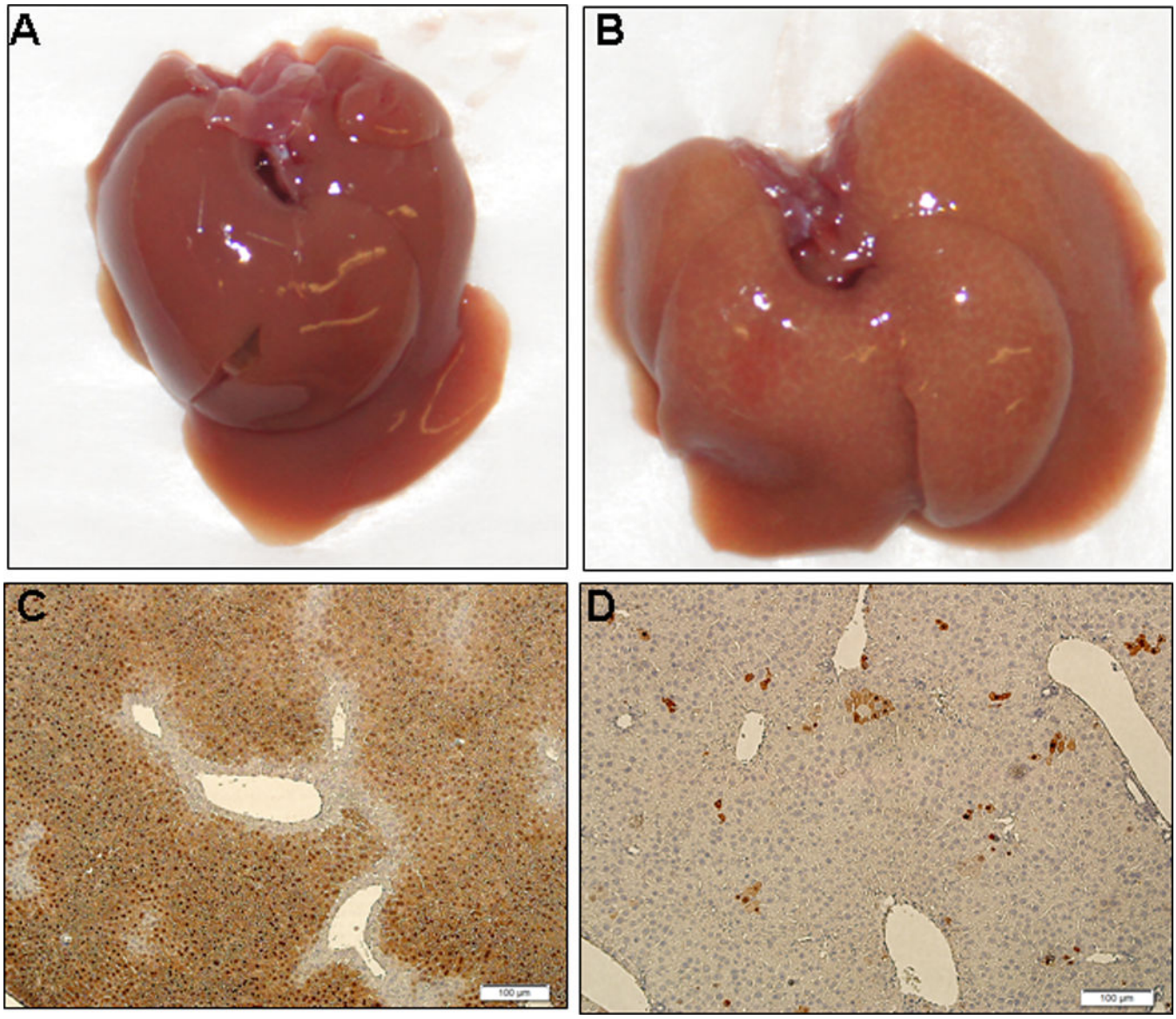


**Figure 2. Gross and microscopic examination of murine liver**

On the left (A) is a liver from a littermate control animal at 4 months of age. On the right (B) is the liver from an AAV-treated ARG1$^{-/-}$ mouse demonstrating the typical appearance of this organ upon removal from the animal. Representative sections of arginase expression in (C) littermate controls and (D) AAV-treated ARG1$^{-/-}$ mice at 4 months of age are depicted. (Both microscopic images are at 100× magnification.)

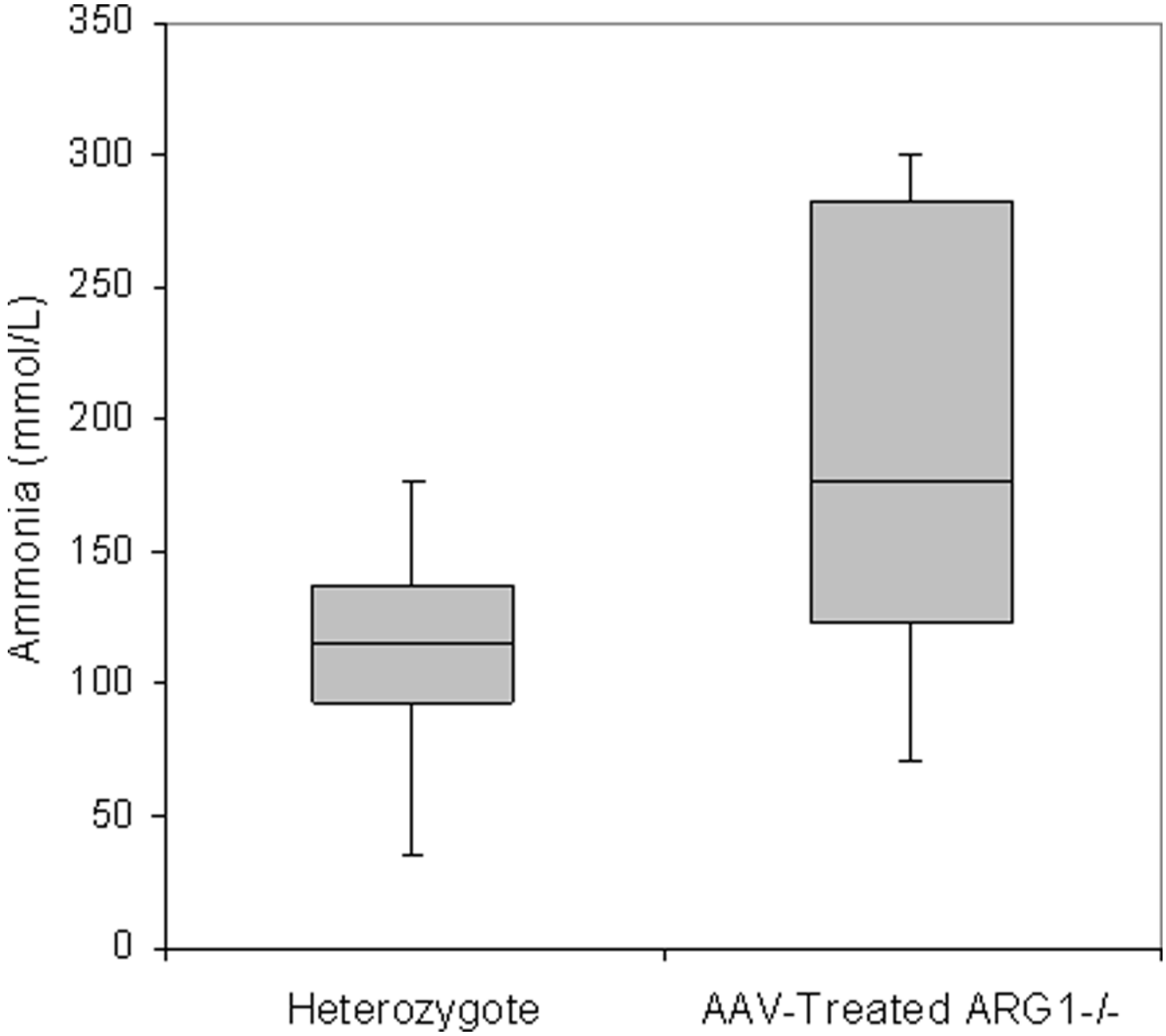


**Figure 3. Plasma ammonia comparison between littermates and AAV-treated ARG1$^{-/-}$ mice**

Plasma ammonia was measured after collecting blood at 4 months in both littermates (n=10) and in AAV-treated animals (n=9). The horizontal line is the box is the median and the horizontal line at the top of the box is the 75$^{th}$ percentile while the horizontal line at the bottom is the 25$^{th}$ percentile. The horizontal lines above and below the box represent the maximum and minimum values.

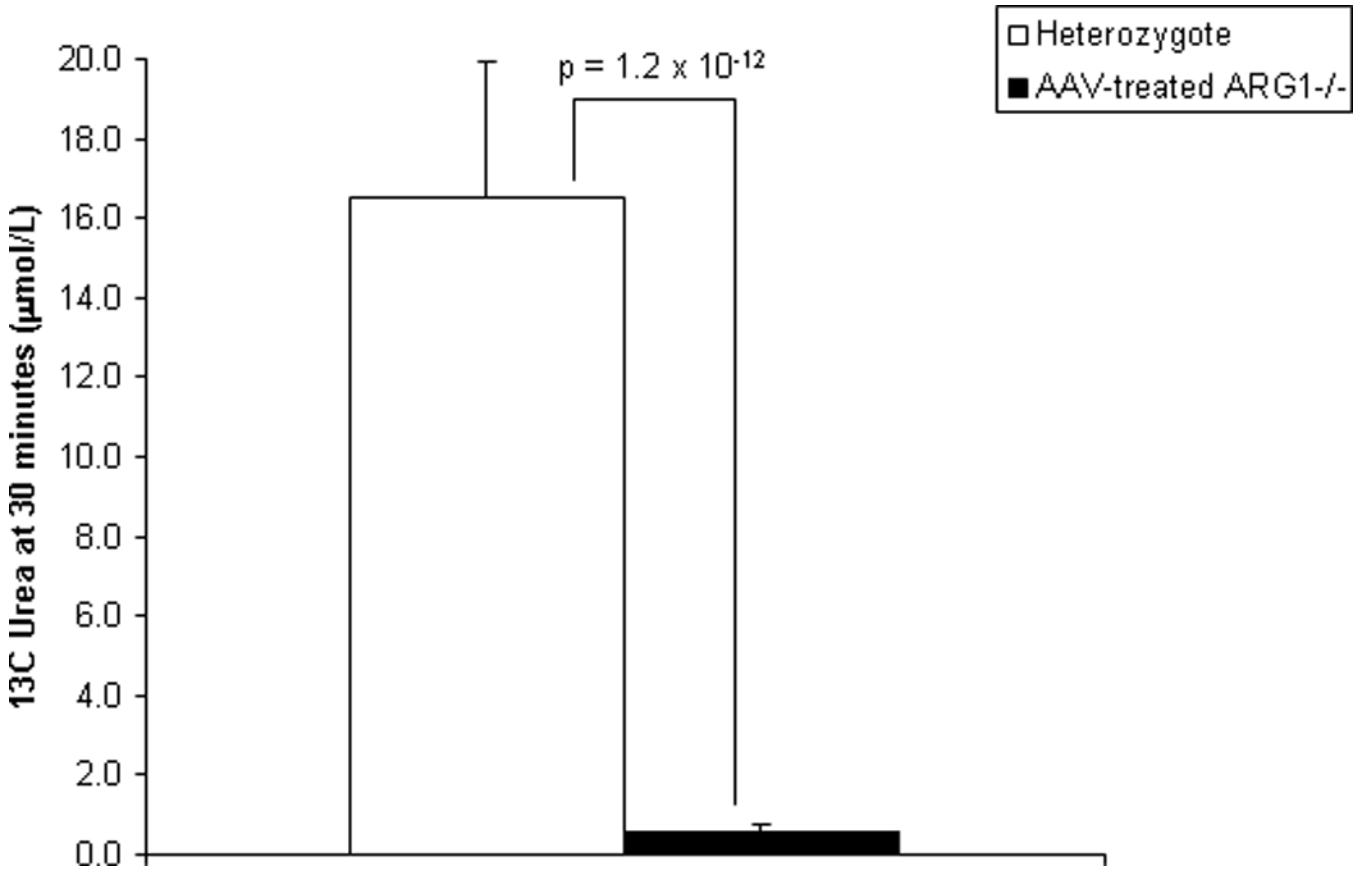


**Figure 4. Ureagenesis in stable adult AAV-ARG1$^{-/-}$ mice and littermate controls**

[$^{13}$C] urea was measured 30 minutes after administration of [1-$^{13}$C] acetate. Adult AAV-treated ARG1$^{-/-}$ mice (n=11) have ureagenesis, albeit at greatly reduced levels compared to littermates (n=11). Error bars represent standard deviation.

**Table 1**

Urea Cycle Amino Acids in Study Groups

| | | Littermate | AAV-Treated Arg1$^{-/-}$ | *p* value |
|---|---|---|---|---|
| | ***Time 0*** | 455.4 ± 121.5 | 921.0 ± 298.3 | 0.0001 |
| **Glutamine** | ***Time 30*** | 357 ± 75.0 | 680.0 ± 216.5 | 0.0001 |
| | ***% change*** | −21.6 | −26.2 | |
| | ***Time 0*** | 71.9 ± 36.9 | 35.8 ± 18.7 | 0.02 |
| **Ornithine** | ***Time 30*** | 59.2 ± 33.4 | 34.5 ± 30.9 | 0.32 |
| | ***% change*** | −17.7 | −3.8 | |
| | ***Time 0*** | 198.3 ± 80.1 | 535.1 ± 129.3 | 4.30E-07 |
| **Arginine** | ***Time 30*** | 154.6 ± 60.8 | 553.4 ± 122.2 | 5.30E-09 |
| | ***% change*** | −22.1 | 3.4 | |
| | ***Time 0*** | 72.9 ± 24.5 | 132.0 ± 39.0 | 0.0004 |
| **Citrulline** | ***Time 30*** | 57.0 ± 20.9 | 143.7 ± 44.2 | 0.00001 |
| | ***% change*** | −21.8 | 8.9 | |

Data are presented as nmol/ml as mean ± SD.