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Liver-Directed Adeno-Associated Virus Serotype 8 Gene Transfer Rescues a Lethal Murine Model of Citrullinemia Type 1

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Abstract

Citrullinemia type 1 (CTLN1) is an autosomal recessive disorder of metabolism caused by a deficiency of argininosuccinate synthetase. Despite optimal management, CTLN1 patients still suffer from lethal metabolic instability and experience life threatening episodes of acute hyperammonemia. A murine model of CTLN1 (*fold/fold*) that displays lethality within the first 21 days of life was used to determine the efficacy of adeno-associated viral (AAV) gene transfer as a potential therapy. An AAV serotype 8 (AAV8) vector was engineered to express the human *ASS1* cDNA under the control of a liver-specific promoter (thyroxine binding globulin, TBG), AAV8-TBG-*hASS1*, and delivered to 7–10 day old mice via intraperitoneal injection. Greater than 95% of the mice were rescued from lethality and survival was extended beyond 100 days after receiving a single dose of vector. AAV8-TBG-*hASS1* treatment resulted in liver specific expression of *hASS1*, increased ASS1 enzyme activity, reduction in plasma ammonia and citrulline concentrations, and significant phenotypic improvement of the *fold/fold* growth and skin phenotypes. These experiments highlight a gene transfer approach using AAV8 vector for liver targeted gene therapy that could serve as a treatment for CTLN1.

Keywords

urea cycle disorders; citrullinemia; AAV8; hyperammonemia

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Citrullinemia type I (CTLN1, OMIM 215700) is an autosomal recessive disorder and results from a deficiency of argininosuccinate synthetase (ASS1), the third enzyme in the urea cycle. The incidence of CTLN1 is estimated to be 1 in 57,000[1]. This urea cycle disorder is characterized by intermittent hyperammonemia and persistent citrullinemia. The clinical spectrum of CTLN1 ranges from neonatal hyperammonemia and death to milder late-onset forms [2]. Due to its relatively recent addition to the newborn screening panel, patients with citrullinemia will be identified early, allowing immediate implementation of treatment. However, despite this early identification of disease and treatment, some patients may progress. The untreated mortality rate in untreated classical CTLN1 is 100%, with most deaths occurring before 17 days of life [3].

Currently the main treatment for patients with CTLN1 involves restriction of dietary protein intake, combined with the addition of the urea cycle intermediate arginine. Liver transplantation has been performed in an attempt to improve metabolic stability through the provision of organ-specific enzymatic activity [4]. Although this approach has been effective, and even curative, for other metabolic disorders, the clinical utility of solid organ transplantation as a standard treatment in all UCD is unclear. While liver transplantation has been successful in lowering hyperammonemia and citrulline in CTLN1, extrahepatic effects can still be seen in persistent renal ASS1 deficiency necessitating continued arginine supplementation [5]. In addition, extrahepatic effects of ASS1 deficiency post transplantation such as NO generation remain to be explored [6, 7]. Overall, the number of cases of liver transplantation for CTLN1 is small, and long-term follow up is not available making it difficult for meaningful conclusions to be drawn regarding the efficacy of this treatment [8–10].

A bovine model of CTLN1 has been described [11]; however, the logistics of generating large numbers of animals for studies and the feasibility of genetic manipulation is difficult in a large animal model. The first mouse model of CTLN1 was generated by a targeted disruption of the *ASS1* gene and termed the *ASS1 KO* mouse [12]. Homozygous mutants develop grossly elevated plasma citrulline levels and hyperammonemia, resulting in death by 48 hours. This life span can be extended up to 6 days with dietary treatment, arginine supplementation and nitrogen scavenging with sodium benzoate. This marginally improved survival occurs in the absence of weight gain.

Recently, two independent hypomorphic recessive mutations at the mouse *ASS1* locus, barthez (*bar*) and follicular dystrophy (*fold*), have been reported [7]. The *fold* allele harbors a T389I substitution in exon 15 leading to an unstable protein structure with normal *ASS1* mRNA and protein levels. Unlike the *ASS1* KO model, *fold* mice survive up to 3 weeks or longer, have 5–10% enzyme activity and display clinical and biochemical parameters similar to CTLN1. In addition, these mice display significant brain abnormalities including defects in neuronal migration and reduced generation of nitric oxide. Since the average length of time to diagnosis of CTLN1 in patients can be up to 1 week or longer (McGuire et al., submitted) the homozygous *fold* mouse is the ideal candidate model for liver targeted gene therapy for the correction of ASS1 deficiency outside the 24–48 hour time period.

The efficacy of gene delivery has been demonstrated in the *ASS1* KO mouse using an E1 deleted adenoviral vector, which carried the *ASS1* cDNA driven by a ubiquitous CMV promoter [13]. Although the adenoviral treated mice did exhibit a modest increase in survival and metabolic improvement, mice treated twice with the adenovirus and supplemented with sodium benzoate survived on average, 40 days. Recent successes in the *spf-ash* mouse, a model of ornithine transcarbamylase deficiency, using adeno-associated viral vectors capable of long-term transgene expression suggests that gene therapy for CTLN1 could be advanced to the clinic [14–16].

Herein, we describe the therapeutic efficacy of a liver targeted single-strand AAV8 vector as a new gene therapy treatment for CTLN1 with the potential for translation to the clinic. The AAV8 treated homozygous *fold* mice are rescued from lethality, display reduced circulating metabolites, and increased hepatic ASS enzyme activity. However, liver directed gene therapy did not fully correct the biochemical phenotype of systemic ASS1 deficiency as arginine levels plummeted in treated *fold/fold* mice due to persistent a renal deficiency. Our results provide the first evidence of the utility of systemic gene delivery for citrullinemia using AAV8.

RESULTS AND DISCUSSSION

Unlike the adenovirus that used a ubiquitous CMV promoter for correction of the ASS1 KO mouse [13], our AAV vector utilized a TBG promoter to direct ASS1 expression to the liver. A total of 20 *ASS1*$^{fold/fold}$ (*fold/fold*) mice received an intraperitoneal injection of 1×10^{10} genome copies (GC) of AAV8 carrying the human *ASS1* cDNA (AAV8-hASS1) under the control of the liver specific TBG promoter at 7–10 days of life. This time point was chosen due to the ease of phenotypic identification of animals (i.e. fur abnormalities) and agreement with time to diagnosis in CTLN1. The AAV8 dose was based on previous descriptions of gene therapy mediated rescue of murine models of methylmalonic and propionic acidemia [17, 18]. All WT mice survived for the duration of study for up to 106 days (N=25). Untreated *fold/fold* mice (N=6) could be recognized at 7–10 days of life but were not recovered at weaning at 3–4 weeks, consistent with the original description of this model. Nearly all (N=19) of the treated *fold/fold* mice (95%) survived the early lethality period (<28 days) up to 106 days ($P < 0.01$, Figure 1A), with the exception of a single treated *fold/fold* mouse that died at 28 days. AAV8 gene therapy significantly prolonged survival when compared to the *ASS1* mouse [13], likely due to the ability of AAV vectors to provide long-term transgene expression in comparison to E1 deleted adenoviral vectors [19].

Fold/fold mice were not distinguishable from *fold/+* or WT littermates at birth. As untreated *fold/fold* mice develop over time, a growth disparity becomes apparent in the rate of weight gain when compared to WT littermates. In a cross sectional analysis of weights, *fold/fold* mice treated with AAV8-h*ASS1* followed a distinct growth curve (Figure 1B) similar to treated *ASS1 KO* mice [13], albeit with greater weight gain. At ~ 80–90 days, *fold/fold* mice (mean = 13.5 g, SD = 2.1) weighed approximately 40% less than littermate controls (mean = 21.6 g, SD = 0.7). In addition to weight, overall length was also decreased compared to WT littermates (Figure 1C). *Fold/fold* was initially described for its abnormal patchy hair pattern

(fold – follicular dystrophy) [7]. With hepatic ASS1 correction, coat texture and fullness was still patchy at 26 days but significantly improved by 52 days.

With improvement in survival of treated *fold/fold* mice, we next looked at the liver 30 days post treatment to examine whether *hASS1* mRNA was expressed and if hepatic ASS1 enzyme activity was increased. Using quantitative PCR, AAV-*hASS1* vector was detectable on average at 567 copies/haploid genome in the treated animals, but was undetectable in WT and untreated animals (Figure 2A). Similar to the vector copy number, treated *fold/fold* demonstrated *ASS1* mRNA levels 35X (SD = 25.6) above WT baseline in most cases (Figure 2B). With the presence of vector and mRNA expression, hepatic enzyme activity was detectable (mean = 5.0, SD = 6.0) in some treated *fold/fold* at 30 days post injection, however this finding was not significant (P = 0.51, Figure 2C). Since pieces of liver were used for the various assays, these results suggest that liver correction was not uniform.

With longer survival in treated *fold/fold*, we next measured plasma ammonia and amino acids to assess biochemical improvement (Figure 2D-F). Similar to the *ASS1* KO mouse [13], the average plasma ammonia and citrulline levels were grossly elevated in untreated *fold/fold.* Blood samples taken approximately 1 month after treatment with gene therapy in *fold/fold* showed a reduction in plasma ammonia levels by > 50% in treated animals (mean = 195.4 μmol/L, SD = 173.2, P = 0.03, Figure 2D).

As with ammonia, plasma citrulline levels were also reduced (Figure 2E). At baseline, plasma citrulline was grossly elevated (mean = 2754 μmol/L, SD = 111.9) in untreated *fold/fold* when compared to WT mice (mean = 110.9 μmol/L, SD = 22.5). Post gene therapy, plasma citrulline levels were reduced by 73% (mean = 756.3 μmol/L, SD = 844.0, P = 0.01) when compared to untreated *fold/fold*, although these levels still remained elevated above WT. Similar to the *ASS1* KO mouse [13], the average plasma citrulline levels remained elevated in *fold/fold.* This variability may be due to stochastic effects due to injection, transduction or expression of the human mRNA.

The kidney plays an important role in the maintenance of plasma arginine levels by converting citrulline to arginine through ASS1 and ASL. This is known as the intestinal-renal axis[1, 20]. Patients with CTLN1 may display arginine deficiency on plasma amino acid profiles due to renal ASS1 enzyme deficiency. In addition, arginine supplementation is often required after liver transplantation for CTLN1 [5]. Surprisingly, pretreatment plasma arginine levels in *fold/fold* were two times greater than WT (mean = 230.0 μmol/L, SD = 9.6, P < 0.01, Figure 2F). Original descriptions of the *fold/fold* model reported mean plasma arginine levels of 171 μmol/L, SD = 68, which can overlap with WT mice [7]. We hypothesized that plasma arginine in *fold/fold* may be related to the large citrulline pool being fed through the hypomorphic ASS enzyme in the kidney, however, these data reflect a specific point in time and may not reflect overall arginine status. More importantly, with liver-targeted gene therapy, *fold/fold* plasma arginine levels plummeted on average to less than half of WT (mean = 26.8 μmol/L, SD = 22.0, P < 0.01) consistent with persistent renal ASS enzyme deficiency seen post liver transplantation [5]. Regarding the *ASS1 KO* post gene therapy, marginal improvement was seen in plasma arginine [13]. This discrepancy in plasma arginine levels between the treated *fold/fold* and ASS1 KO levels is likely related to the vectors used.

In the *ASS1 KO*, the adenoviral vector had a ubiquitous promoter, which may transduce the kidney and result in an improved intestinal-renal axis.

In conclusion, these studies are the first to establish the efficacy of liver directed gene therapy in the rescue of argininosuccinate synthetase deficiency using AAV8. Improvements in biochemical parameters and survival are clearly demonstrated. In addition, the importance of extrahepatic ASS1 deficiency in the maintenance of plasma arginine levels is suggested by the metabolic parameters observed in the treated *fold/fold* mice.

METHODS

Murine model of CTLN1

The *fold* mutation was initially described at the Jackson Laboratory in a production colony of P/J mice. Congenic (N4) B6Ei.P-*fold*/J (stock number 006449) mice were purchased from The Jackson Laboratory (Bar Harbor, ME); herein referred to as *fold*. *Fold* mice display elevated plasma ammonia and massively elevated plasma citrulline concentrations. Wild-type (WT) littermate animals were used as controls throughout.

AAV8 construction, production, and delivery

The expression vector, pENN-AAV-TBG-PI-RBG (PennVector P1015) was obtained from the University of Pennsylvania Vector Core. This vector contains transcriptional control elements from the thyroid binding globulin (TBG) promoter, cloning sites for the insertion of a complementary DNA, and the rabbit β-globin polyA signal. Terminal repeats from AAV serotype 2 flank the expression cassette. The human *ASS1* cDNA was isolated from a human liver cDNA library by RT-PCR using *ASS1* specific primers (listed below) and was sequence verified. The *ASS1 cDNA* was then cloned into pENN-AAV-TBG-PI-RBG. This newly created vector AAV-TBG-PI-ASS1-RBG was packaged into AAV8, purified by cesium chloride centrifugation, and titered by qPCR as previously described [21]. Animal studies were reviewed and approved by the National Human Genome Research Institute Animal Care and Use Committee. Viral particles were diluted to a total volume of 100 microliters with phosphate-buffered saline immediately before injection and 1×10^{10} genome copies (GC) were administered via intraperitoneal injection at 7–10 days of age.

ASS1-Koz-XhoI CTCGAGgccaccATGTCCAGCAAAGGCTCCGTG

ASS1-Stop-MluI acgcgtCGGGTCTATTTGGCAGTGAC

Quantitatification of hepatic ASS1 mRNA expression

Total RNA was extracted from the liver using RNeasy Mini Kit (Qiagen, Valencia, CA), and DNase digested was preformed using DNA-free (Ambion, Austin, TX). Reverse transcription was preformed using Applied Biosystems High capacity cDNA Transcription Kit. Quantitative real-time PCR was subsequently preformed on the cDNA with TaqMan gene expression assays [murine β-actin (Mm00607939_s1) and murine *Ass1* (Hs01597989_g1) from Applied Biosystems, Foster City, CA]. Samples were analyzed in an Applied Biosystems 7500 fast real-time PCR system, in accordance with the manufacturer's protocol. All samples were analyzed in triplicate.

Vector genome copy number

Genome copy (GC) number was measured by quantitative real-time PCR analysis. A standard curve was prepared, using serial dilutions of the AAV plasmid carrying AAS1 cDNA. Genomic DNA was extracted from murine liver samples and murine genomic DNA was used to determine the vector genome copy number per mouse haploid genome.

Metabolic studies

Plasma was isolated from blood collected by retro-orbital bleeding. The samples were immediately centrifuged, and the plasma was removed and stored at −80 °C for later analysis. Plasma citrulline and arginine were analyzed by ion exchange chromatography (Biochrom 30, Holliston, MA). ASS1 activity was determined using a clinically available enzyme assay which measures the conversion of ^{14}C-aspartate into argininosuccinic acid[22] (Clinical Biochemical Genetics, Baylor College of Medicine, Houston, TX).

Statistical analyses

In all instances, *P* values were considered significant if the value was <0.05. Kaplan-Meier survival curves were used to compare groups on the basis of treatment with or without liver targeted gene replacement therapy. The weights between treated and untreated mice, and differences in metabolite and enzyme levels were assessed using a two-sided, two-tailed unpaired Student's *t*-test.

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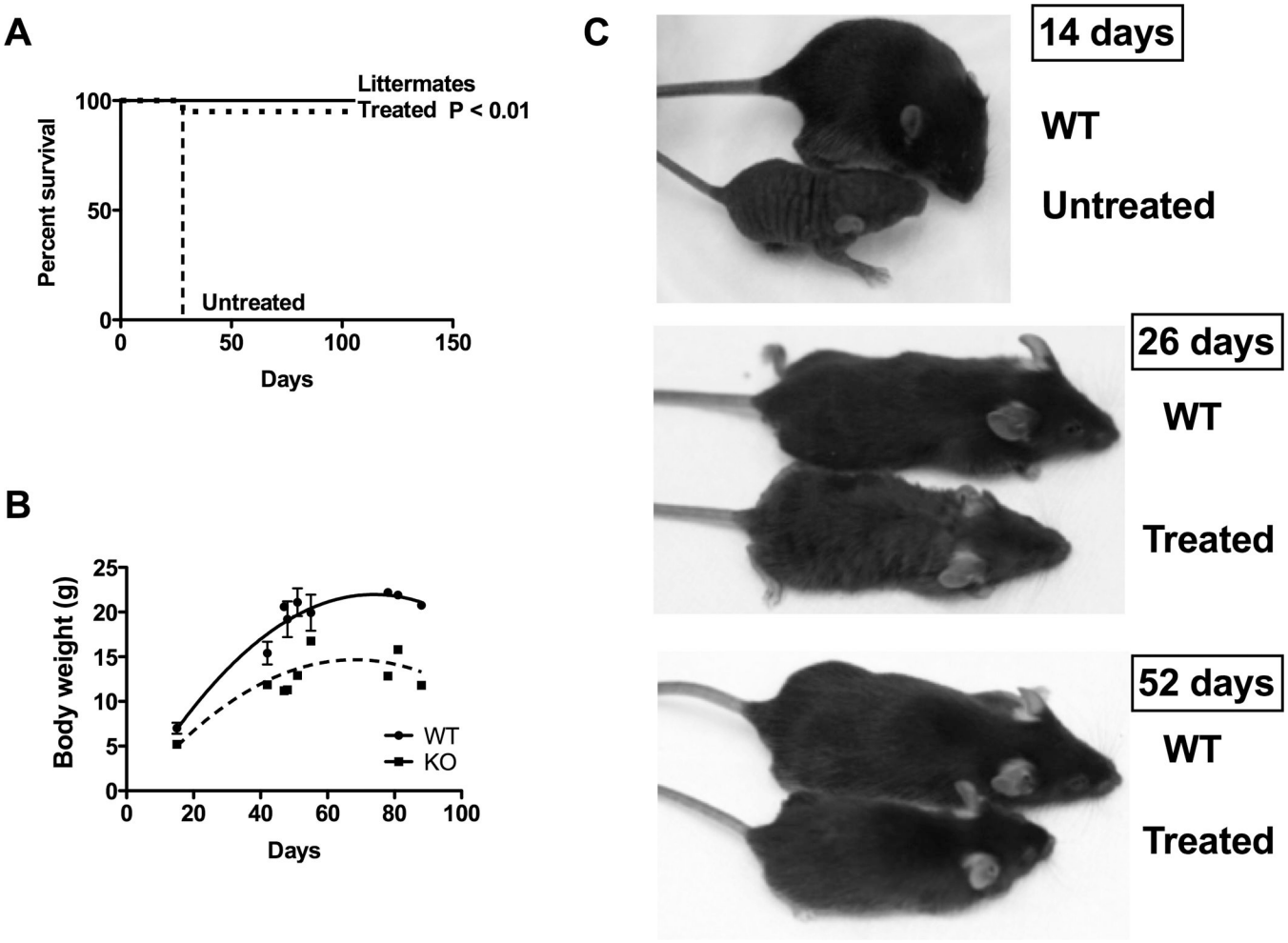

Figure 1.

Survival, weight, length and coat in *fold/fold* treated with liver targeted gene therapy. *Fold/fold* mice received 1×10^{10} GC/mouse at 7–10 days of life. (A) Survival in untreated *fold/fold* (N=6), treated *fold/fold* (N=20) and WT littermates (N=25). (B) Cross-sectional analysis of weights in *fold/fold* (N=19) and WT littermates (N=20). (C) Length and coat texture in untreated *fold/fold*, treated *fold/fold* and WT littermates.

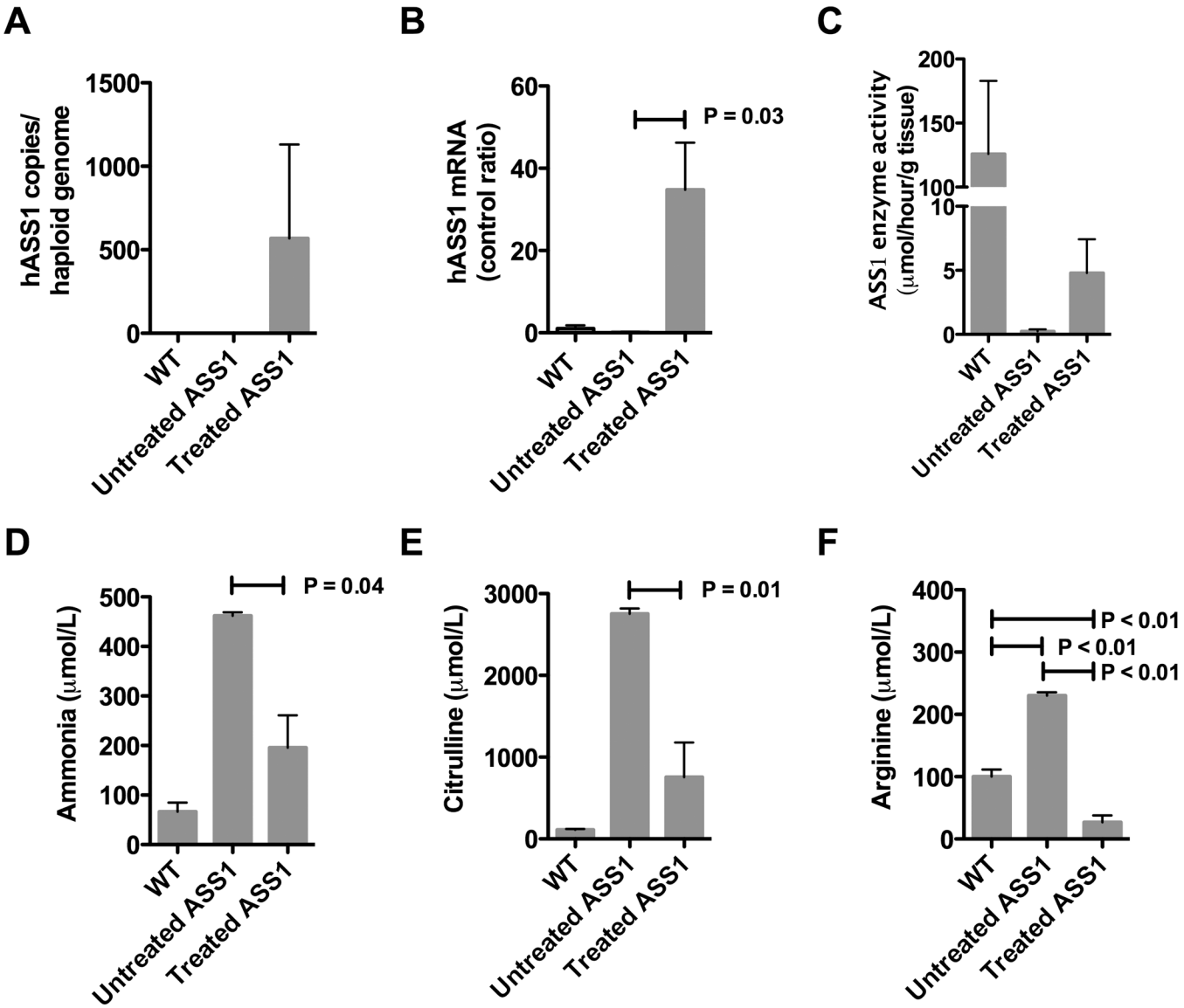

Figure 2.

Hepatic correction in treated *fold/fold* 1 month post gene therapy. (A) Quantitative PCR detection of vector copy number/haploid genome in WT (N=3), untreated (N=3) and treated *fold/fold* (N=6). Data is represented as a ratio to WT levels (control ratio). (B) RT-qPCR detection of hASS1 mRNA in WT (N=3) and treated *fold/fold* (N=3). (C) Enzyme activity in WT (N=3), untreated *fold/fold* (N=3) and treated *fold/fold* (N=5) 1 month post gene therapy. (D) Plasma ammonia was measured in WT littermates (N=4), untreated *fold/fold* (N=3), and treated *fold/fold* (N=7). (E) Plasma citrulline was measured in WT littermates (N=4), untreated *fold/fold* (N=3), and treated *fold/fold* (N=4). (F) Plasma arginine was measured in WT littermates (N=4), untreated *fold/fold* (N=3), and treated *fold/fold* (N=4). Hatched bars indicate $p < 0.05$