Molecular Therapy
Nucleic Acids
Commentary

An oversized AAV8 vector to deliver CPS1

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https://doi.org/10.1016/j.omtn.2025.102504

Carbamoyl phosphate synthetase 1 (CPS1) deficiency is a severe urea cycle disorder that can lead to life-threatening hyperammonemia, coma, and death if left untreated. The prevalence of this condition ranges from 1 in 300,000 to 1 in 1.3 million births, although estimates may vary due to the challenges in diagnosis.[1] Current therapeutic options, such as CarbaGlu, an FDA-approved treatment for N-acetylglutamate synthase (NAGS) deficiency, only benefit a subset of CPS1 patients with specific mutations.[2] Patients with complete CPS1 protein loss require alternative treatments to mitigate the disorder's severe complications.

A recent study published in *Molecular Therapy Nucleic Acids* by Diep et al. presents a promising therapeutic strategy using an oversized adeno-associated virus (AAV) vector to address CPS1 deficiency.[3] This study demonstrated long-term survival with ammonia control in *Cps1*-deficient mice treated with AAV8.CPS1, while all null vector-injected controls succumbed to hyperammonemia.

Given the large cDNA size of *CPS1* (4.5 kb), one of the primary challenges in developing an AAV-based therapy has been the vector's limited packaging capacity (~4.7 kb). To control the overall genome size, the researchers kept all regulatory components as small as possible. The final genome was minimized to 5,263 bp from inverted terminal repeat (ITR) to ITR and packaged into AAV8 for *in vivo* studies (Figure 1).

Using high-fidelity genome analysis, Diep et al. observed a distinct peak in read distribution between 4.97 and 5.28 kb, aligning with the expected size of AAV8.CPS1. The study found that the predominant genomic form was fragmented AAV8.CPS1 ("partial single-stranded AAV [ssAAV]"), accounting for 63.01% of the total. In contrast, full-length ssAAV ("full ssAAV") was scarce, representing only 0.49%. Notably, almost no vector sequences exceeded 5.2 kb, suggesting that the upper packaging limit for AAV8 is approximately 5.2 kb, consistent with other previous reports.

In this study, $Cps1^{flox/flox}$ mice at 2 months of age were intravenously administered AAV8 expressing Cre recombinase together with either AAV8.CPS1 or AAV8.null vector at a dose of 3E+14 gc/kg, approximately 30-fold higher than that used for AAV8.TBG.Cre. The treated mice exhibited prolonged survival (up to 9 months) with stable ammonia levels, while control mice died due to hyperammonemia by day 27–36. The AAV8.CPS1-treated animals maintained their body weight and exhibited normalized plasma alanine aminotransferase (ALT) levels without detectable liver pathology. Ureagenesis was also partially restored, as evidenced by a metabolic response to an ammonium challenge.

Interestingly, female $Cps1^{flox/flox}$ mice responded better to AAV8.CPS1 gene therapy, with a significantly higher survival rate compared to their male counterparts. This trend is consistent with an earlier study by the same group using an overlapping dual vector approach.[4] The underlying factors contributing to the gender differences in response to CPS1 gene delivery in this mouse model remain unclear and warrant further investigation. Additionally, it is unclear whether the oversized AAV strategy offers any advantages over the previously reported overlapping dual vector approach for CPS1 delivery, as the authors did not provide a direct comparison between these two approaches. A comparative analysis would be beneficial to determine the most effective method for CPS1 gene therapy.

Despite the promising results, the high percentage of fragmented genomes (63.01%) observed in this study raises concerns about genotoxicity, particularly when high doses are required. To address these concerns, a thorough evaluation of the safety profile in small and large animal models is necessary before advancing to clinical testing. Furthermore, the need to expand the packaging capacity of AAV is highlighted by this and other studies. Several strategies, including fragmented genome assembly, overlapping dual vector, *trans*-splicing dual vector, and hybrid approaches combining these, have shown promise in delivering large genetic payloads. However, their efficiency needs to be improved to maximize therapeutic outcomes at a safer, lower dose. Recently, split-intein-mediated protein *trans*-splicing has been successfully employed in dual and triple vectors to deliver base editors, midi-dystrophin, and even full-length dystrophin,[5–7] presenting a promising solution for delivering large-sized genetic medicine using AAV. Additionally, human bocavirus has been found to be capable of packaging the AAV genome, offering an alternative solution for gene therapy with its 5.5 kb packaging capacity.[8] Finally, machine-learning-assisted capsid engineering may provide critical insights into overcoming the size constraint of AAV, potentially revolutionizing the field of gene therapy. By exploring these innovative approaches, researchers can work toward developing safer and more efficient methods for delivering large genetic payloads, ultimately improving therapeutic outcomes for patients.

In summary, the study by Diep et al. demonstrates the potential of using an oversized AAV8 vector to treat CPS1 deficiency, supporting the feasibility of AAV-based CPS1 gene therapy. However, challenges such as AAV genome fragmentation, a low

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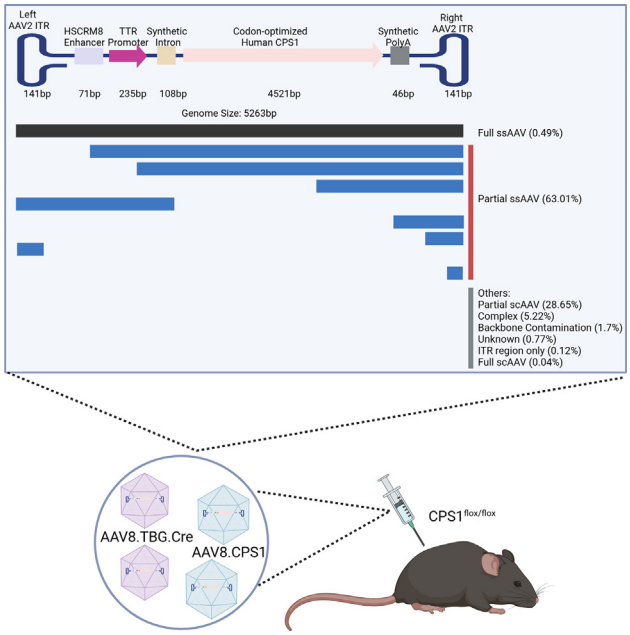

Figure 1. Schematic illustration of the oversized AAV8.CPS1 vector and fragmented genome
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percentage of full particles, and sex-specific differences in therapeutic responses underscore the need for further investigation.

ACKNOWLEDGMENTS

R.H. is supported by US National Institutes of Health grants (R01HL169976, R01HL159900, R01HL170260, and R21HL163720).

DECLARATION OF INTERESTS

R.H. is a section editor of *Molecular Therapy* and a founder of Zhida Therapeutics with interests in developing gene therapies. R.H. is an inventor of patent applications (63/625,504, 63/421,383, and PCT/US2021/017868) related to gene therapies.

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