



Comparing Gene Therapy Methods as Permanent Treatments for Phenylketonuria PKU

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Abstract

Phenylketonuria (PKU) is a genetic disorder resulting from a deficiency of phenylalanine hydroxylase (PAH), which leads to the accumulation of the harmful amino acid phenylalanine. Current treatment includes a strict diet, effective for all patients. However, patients often struggle to maintain their diet, so they seek a permanent solution or flexibility in their eating habits. FDA-approved medicine Kuvan can help patients have a more flexible diet. Unfortunately, Kuvan has severe side effects; it is not permanent, and it can't help all PKU genotypes adapt to their diets. A permanent solution can be achieved through the use of gene therapy methods. Gene therapy can be used in finding permanent treatments for genetic disorders. Gene therapy offers a potential cure by targeting the genetic cause. Methods in gene therapy, such as recombinant adeno-associated viral (rAAV), naked DNA, and CRISPR/Cas9 gene editing, were tested on PKU lab-made mice to treat PKU. (Grisch-Chan, Schwank, Harding, & Thöny, 2019, p. 1277) This study evaluates rAAV, naked DNA, and CRISPR/Cas9 based on their ability to lower Phenylalanine (Phe) levels, their long-term stability, and their safety profiles in mouse models. The analysis indicates that while rAAV vectors achieve significant initial Phe reduction, the effect is often transient due to episomal dilution. Naked DNA demonstrates safety in mice but remains unscalable due to the high-pressure delivery methods required. CRISPR/Cas9 offers the most precise genomic correction, yet current trials show limited editing efficiency in the total liver cell population. Ultimately, while no method currently provides a perfect cure, CRISPR/Cas9 gene editing is identified as the most promising candidate for permanent treatment as it corrects the underlying problem of the genes, while still needing to resolve safety concerns such as off-target effects.

Keywords: PKU, Kuvan, gene therapy, treatment

I. Introduction

PKU (Phenylketonuria) is an autosomal recessive genetic disorder caused by the deficiency of the enzyme Phenylalanine Hydroxylase (PAH), which converts phenylalanine to tyrosine in the liver (Brown & Lichter-Konecki, 2016, p. 9). Tyrosine is another amino acid transported through the bloodstream to the brain and is essential for synthesizing neurotransmitters involved in cognitive function and neurological development. In this disease, patients experience an excess of phenylalanine and an insufficient amount of tyrosine in their bodies, leading to neurological, psychological, and physical problems. Patients need to follow a strict low-protein diet to prevent these consequences from occurring. A strict regimen must be followed for the rest of the patient's life, making it a lifelong but non-curative treatment. To find a permanent treatment, sapropterin dihydrochloride, also known as Kuvan, was developed and received approval from the Food and Drug Administration (FDA). Kuvan represents a significant advancement for PKU patients, as it supplies tetrahydrobiopterin (BH4), a non-protein chemical compound that acts as a cofactor for PAH, thereby enhancing the

enzyme's residual catalytic activity. However, it is only effective in patients who retain some functional PAH protein, since the cofactor can stabilize and enhance residual activity but cannot compensate for mutations that eliminate enzyme function. As a result, Kuvan benefits only a subset of patients, causing side effects in most, and does not correct the underlying genetic mutation that causes the disease, which means it is a temporary treatment. A permanent solution that can eliminate the need for a specialized diet and solve the problem of PKU patients once and for all is gene therapy. Gene therapy is advancing and improving every day, helping scientists cure mutations. Gene therapy, which targets the root genetic cause of PKU by correcting mutations in the PAH gene on chromosome 12, has shown promising potential. Its clinical success in other genetic disorders, such as spinal muscular atrophy (SMA), demonstrates the feasibility of this approach in treating inherited diseases. However, PKU presents a unique challenge for gene therapy due to its vast mutational landscape—over 1,000 different PAH mutations have been identified—making it a rigorous test case for developing versatile and mutation-agnostic therapeutic approaches. This proposal aims to compare the experiments made using gene therapy methods to assess their potential as a permanent treatment for the genetic disorder PKU.

II. Literature Review

Conventional therapies such as dietary restriction and the use of sapropterin (Kuvan) have shown limited and inconsistent success, leaving a clear need for permanent solutions. Gene therapy offers an alternative approach by directly targeting the root cause of PKU — mutations in the PAH gene. In recent years, adeno-associated virus (AAV) vectors have been investigated as delivery tools to restore PAH activity in the liver, with several studies in PKU mouse models showing long-term correction of elevated phenylalanine levels. These approaches aim not only to normalize metabolism but also to overcome the lifelong burden of dietary therapy. Additionally, Kuvan can have critical side effects and risks. As mentioned in the FDA report, prolonged elevations in blood Phe levels in patients with PKU can result in severe neurologic damage, highlighting the risks of improper or ineffective use. The most serious adverse reactions during Kuvan administration are gastritis, streptococcal infection, and urinary tract infection. Mild to moderate neutropenia, which means a lower number of white blood cells than normal, was noted during Kuvan administration in 24 of 579 patients (4%) (U.S. Food and Drug Administration, 2007). The most frequently reported side effects of Kuvan ($\geq 4\%$ of patients; $n=579$) were headache, nausea, vomiting, abdominal pain, diarrhea, and mild upper respiratory tract infections (U.S. Food and Drug Administration, 2007). More severe but less common adverse reactions, such as pharyngolaryngeal pain, have also been documented. Kuvan, showing varying effects in patients and not correcting the underlying genes, makes it a temporary, non-universal treatment.

If not adhering to the diet or left untreated, individuals can face severe complications such as severe intellectual disability, seizures, psychiatric problems, and neurological problems (van Vliet et al., 2019, p. 7). Gene therapy was experimented on “PKU” mouse models. PKU mouse models were created by introducing a specific point mutation in the PAH gene, resulting in severely reduced PAH enzyme activity and elevated phenylalanine levels, closely mimicking human disease. Recent approaches to PKU gene therapy have included several strategies: recombinant adeno-associated viral (rAAV) vectors, which are modified viruses used to deliver healthy PAH genes into cells; nonviral naked DNA vectors, which deliver DNA without a viral carrier; genome editing with base editors, which directly correct mutations at the single-letter level of DNA; and promoter-less PAH-mRNA integration, which inserts new PAH instructions into the natural location of the gene (Grisch-Chan, Schwank, Harding, & Thöny, 2019, p. 1275). Each of these strategies operates distinctly. rAAV vectors use a harmless virus as a carrier to deliver a healthy copy of the PAH gene into liver cells, restoring the ability to break down phenylalanine. Naked DNA vectors attempt the same without using a virus, instead injecting free DNA directly into cells, which works well in mice but is less efficient in larger animals due to the inability to safely replicate high-pressure injection volumes and the rapid enzymatic degradation of unprotected DNA in larger circulatory systems. Base editors are a form of CRISPR technology that directly correct a single-letter mutation in the DNA, offering precision

but requiring customization depending on the patient's mutation. The rAAV experiment was conducted in 2006 and successfully reduced the Phe level in the PKU mouse, although the reduction was not permanent. However, rAAV experiments were stopped due to the virus provoking a profound host immune response. Early attempts at liver-targeted gene therapy using rAAV vectors were not very successful because only a small number of liver cells actually received and expressed the PAH gene. As a result, the treatment could only partially lower blood phenylalanine levels, instead of fully correcting hyperphenylalaninemia. (Grisch-Chan, Schwank, Harding, & Thöny, 2019, p. 1277).

While the hydrodynamic injection method of naked DNA vectors is an effective gene delivery method for hepatocytes in mice, achieving physiologically relevant hepatocyte transduction in larger animals or humans remains a major challenge. Nevertheless, modifications to achieve efficient delivery of naked DNA to the liver upon hydrodynamic injections of large mammals is being explored. (Viecelli et al., 2014, 1042).

The future development of CRISPR/Cas9 gene editing should be designed to be broadly applicable to all human PKU genotypes and should aim to reduce Phe levels to wild-type (WT), normal, and healthy levels (Richards et al., 2020, p. 252). Genome editing is possible and efficient. However, it does not reduce Phe levels to normal levels. Since more than a thousand different mutations in the PAH gene can cause PKU, including missense, nonsense, and splicing variants, gene therapy tools often need to be customized to the patient's specific mutation type. While mutation-specific approaches like base editing may require customization for each patient, full gene replacement using rAAV vectors bypasses this issue by delivering an intact PAH gene, making it independent of the patient's specific mutation.

III. Methods

This study aims to identify the most effective gene therapy method for treating phenylketonuria by comparing experimental results from three approaches of gene therapy methods: recombinant adeno-associated viral (rAAV) vectors, nonviral naked DNA vectors, and gene editing techniques using CRISPR/Cas9. These methods are preferred in PKU experiments for their effectiveness in finding a cure for other diseases like hemophilia. Each of these gene therapy methods was tested in PKU mouse models, which carry a single amino acid substitution in the PAH gene that causes severely diminished PAH catalytic activity. These mutations provide a well-established model of severe Phenylketonuria, closely resembling the condition observed in human patients. After various experiments on mice, differing results were found based on each treatment. In this comparison, the data and charts available in articles published in Google Scholar, FDA reports, and journal articles, which include publishing years from 2014 to 2024, were used. This study aims to find the utmost efficiency, safety, and permanency among these methods. This proposal is written to use the method of comparative literature-based analysis, where the varying experiments will be compared, to answer the question of which procedure should continue to help these people and identify the gaps in experiments and research related to these methods. The data for comparing these methods includes quantitative data and qualitative data provided by scientists from their respective sources. Factors like reduction of phe levels, safety, and the response of the immune system to treatment, duration, and human applicability will be compared and analyzed thoroughly. Statistical outcomes such as mean Phe reduction, percentage normalization, and reported duration in weeks will be extracted from studies. Qualitative outcomes such as safety events and immune response will be coded and compared across methods.

IV. Results

This section will analyze and compare the findings from preclinical studies of three gene therapy methods: recombinant adeno-associated viral (rAAV) vectors, naked DNA vectors, and CRISPR Cas9 gene editing in mice models of phenylketonuria (PKU). Each method is evaluated based on published experimental data, focusing on outcomes related to phenylalanine (Phe) level reduction, duration of effect, delivery efficiency, and safety. Summarized data are presented

below in Tables 1 through 3.

4.1 Data

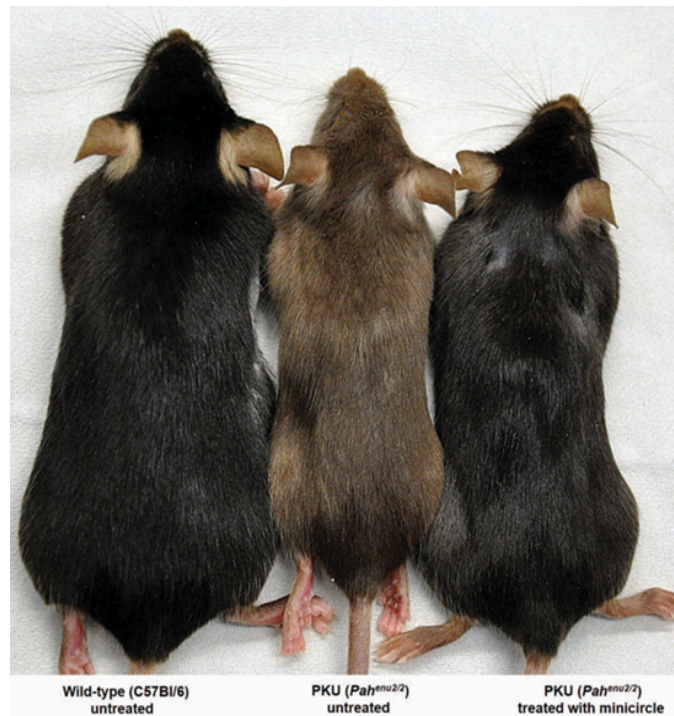


Figure 1. Comparing three mice from the same breed: respectively, from left to right, Mice A being non-PKU, Mice B being untreated PKU, and Mice C being treated PKU. Image taken from Grisch-Chan et al. (2019) medical article.

These studies used mouse models that have PAH deficiency to replicate PKU patients in experiments. Also called Pah^{enu2/2}, these mouse models were injected to have PAH deficiency. Comparisons between treated and untreated mice in the original articles provide visual and experimental evidence of therapy effectiveness. All tables below are adapted from data reported in Grisch-Chan et al. (2019), cited fully in the references section.

4.1.1 Recombinant Adeno-Associated Viral (rAAV) Vector Studies

Table 1 summarizes rAAV-based approaches. Overall, the results demonstrate that improvements in vector design and targeting significantly enhanced outcomes over time. The early rAAV2/2 vector, delivered intraperitoneally, showed poor transduction efficiency and only partial Phe reduction for up to 25 weeks, highlighting the limitations of first-generation vectors. rAAV2/5 improved outcomes, achieving full correction for up to 40 weeks, though at the cost of requiring high doses and showing a sex-dependent effect, with female mice responding less effectively. Later generations, particularly rAAV2/8 and its self-complementary version scAAV2/8, achieved the strongest results. rAAV2/8 restored 100% wild-type PAH activity at high doses. It maintained correction for 42 weeks without toxicity, while scAAV2/8 normalized Phe levels with lower doses and additionally corrected neurotransmitter deficiencies. Finally, rAAV2/1 showed long-term (70-week) Phe normalization when targeted to muscle, though it required coexpression of the cofactor BH4. These results suggest that liver-directed vectors (especially rAAV2/8 and scAAV2/8) provide the most robust and durable correction in PKU mice. In contrast, muscle-directed vectors like rAAV2/1 may offer alternative strategies when combined with BH4 supplementation.

Table 1. Summary of rAAV Vector Studies in PKU Mice

Study/ Vector Type	Injection Method	Phe Reduction	Duration	Efficiency	Safety	Notes
rAAV2/2 (Oh et al.)	Intraperitoneal	Reduced Phe; incomplete correction	Up to 25 weeks	Poor transduction	Not specified	Early generation vector
rAAV2/5 (Mochizuki et al.)	Portal vein	Correction; Phe returned at 40 weeks	Up to 40 weeks	High dose needed; sex-depende nt response	No major toxicity	Female mice are less responsive
rAAV2/8 (Harding et al.)	Portal vein / i.v.	Complete correction in both sexes	Up to 42 weeks	100% WT PAH activity at high dose	No toxicity or anti-PAH antibodies	Best overall performance
scAAV2/8 (Yagi et al.)	Intraperitoneal	Full Phe normalization	Over 1 year	High efficacy at lower dose	Safe	Corrected neurotransmi tter deficiency
rAAV2/1 (Ding et al.)	Muscle	Normalized Phe	Up to 70 weeks	Co-expressio n of BH4 required	Safe	Muscle-targe ted therapy with a triple-cistron ic vector

Note. rAAV = recombinant adeno-associated virus; PKU = phenylketonuria; Phe = phenylalanine; WT = wild-type; PAH = phenylalanine hydroxylase; BH4 = tetrahydrobiopterin; i.v. = intravenous; sc = self-complementary.

4.1.2 Non-viral Naked DNA Vector Studies

Table 2. Summary of Naked DNA Vector Studies

Study/ Vector Type	Injection Method	Phe Reduction	Duration	Efficiency	Safety	Notes
Minicircle DNA (Viecelli et al.)	HTV (hydrodynamic tail vein)	Moderate Phe reduction	Up to 52 weeks	High transduction in mice only	Safe in mice	Delivery method not scalable to humans
mcoPah minicircle (Grisch-Chan et al.)	HTV	Better expression with codon-optimized gene	Up to 60 weeks	Improved over basic minicircles	Safe in mice	Still limited by delivery barriers in larger animals

Note. *HTV* = hydrodynamic tail vein injection; *Phe* = phenylalanine; *mcoPah* = codon-optimized phenylalanine hydroxylase minicircle.

Table 2 summarizes the results of naked DNA vector experiments in PKU mice. These vectors rely on hydrodynamic tail vein (HTV) injection, which floods the liver with DNA but is not feasible in humans due to the large fluid volumes required. In the study by Viecelli et al., minicircle DNA achieved a moderate reduction in phenylalanine levels for up to 52 weeks, demonstrating that the method can provide relatively long-term correction in mice. However, its efficiency was limited to small animals, and the delivery method cannot be scaled to humans. Grisch-Chan et al. tested an optimized version, called *mcoPah* minicircles, which used codon optimization to improve PAH expression. This strategy showed better *Phe* reduction and lasted up to 60 weeks, confirming that the approach can be enhanced at the genetic design level. Both studies confirmed that the method was safe in mice, with no major adverse effects reported. Nevertheless, the main limitation is delivery: while naked DNA vectors are effective in rodents, their translation to larger animals and humans remains impractical due to the inefficiency of uptake and risks associated with HTV delivery.

4.1.3 CRISPR/Cas9 Gene Editing Studies

Table 3. Summary of Gene Editing Studies in PKU

Study/ Vector Type	Delivery Method	Phe Reduction	Duration	Efficiency	Safety	Notes
Richards et al. (2020)	AAV-mediated (i.v.)	Partial reduction	Long-term , persistent correction (up to 65 weeks)	Precise editing, but incomplete correction	No major side effects	Genotype-specific; requires patient customization
Villiger et al. (Grisch-Chan review)	AAV vectors (in vitro/in vivo)	Functional editing in humanized mice	Short-term tested	Targeted insertion at the PAH locus	Not fully assessed	Proof of concept stage

Note. *AAV* = adeno-associated virus; *i.v.* = intravenous; *Phe* = phenylalanine; *PAH* = phenylalanine hydroxylase; *PKU* = phenylketonuria.

Table 3 summarizes experiments using CRISPR/Cas9 gene editing in PKU mouse models. This approach showed high precision in targeting the PAH gene and partially reduced phenylalanine levels for long periods—up to 65 weeks in some cases. Importantly, no major side effects were reported in mice, which supports the potential safety of the method at the preclinical level. Unlike rAAV or naked DNA, CRISPR has the advantage of directly correcting the mutation in the genome, meaning the effect could be permanent if enough cells are edited. However, the current results remain partial because only a subset of liver cells received successful edits. This raises concerns about editing efficiency and whether complete correction can be achieved in larger organisms.

V. Discussion

The comparative analysis of recombinant adeno-associated viral (rAAV) vectors, naked DNA vectors, and CRISPR/Cas9 gene editing reveals distinct therapeutic profiles across five critical domains: efficacy, safety, delivery feasibility, mutation flexibility, and readiness for clinical translation. Each approach offers unique advantages while facing significant barriers that must be addressed before a curative treatment for PKU can be realized.

The rAAV vectors, particularly liver-tropic serotypes such as rAAV2/8 and its self-complementary variant scAAV2/8, demonstrated the most robust and sustained reduction of phenylalanine (Phe) levels in PKU mouse models. Full phenotypic correction was maintained for over one year with scAAV2/8, highlighting the potential for long-term metabolic normalization. In contrast, naked DNA vectors achieved only moderate, though persistent, Phe reduction—up to 60 weeks with codon-optimized constructs—but failed to reach complete correction. CRISPR/Cas9-mediated editing resulted in partial, durable Phe lowering (up to 65 weeks), yet editing efficiency remained insufficient to restore wild-type metabolic function, underscoring a key limitation in current precision gene-editing platforms.

Safety outcomes varied considerably across modalities. rAAV vectors were well-tolerated in mice with no reported toxicity or anti-PAH antibodies; however, immunogenic responses to viral capsids in humans may limit redosing and provoke inflammatory reactions. Naked DNA vectors posed no significant safety concerns in preclinical studies, but the hydrodynamic injection procedure itself carries acute hemodynamic risks that are not clinically feasible. CRISPR/Cas9 raised no major adverse events in mice, yet the potential for off-target genomic alterations and genotoxic consequences in human hepatocytes remains a topic of paramount concern, necessitating comprehensive off-target screening and long-term surveillance in translational studies.

Delivery remains a pivotal translational bottleneck. rAAV benefits from established clinical delivery routes (e.g., intravenous or portal vein infusion) and has been successfully used in FDA-approved gene therapies, though liver-specific targeting and neutralizing antibodies may limit efficacy. Naked DNA delivery via hydrodynamic tail vein injection is effective in rodents but cannot be scaled to humans due to the prohibitive fluid volumes required. CRISPR/Cas9 systems face dual delivery challenges: efficient *in vivo* packaging (often using AAV vectors) and ensuring sufficient editing rates in target tissues without triggering immune clearance or DNA damage responses.

PKU is characterized by extensive allelic heterogeneity, with over 1,000 documented PAH mutations. rAAV and naked DNA vectors are inherently mutation-agnostic, as they deliver a functional PAH cDNA independent of the patient's specific genotype. This gives them broad applicability across the PKU population. In contrast, CRISPR/Cas9 strategies—particularly those using base editors or homology-directed repair—require customization to each mutation or haplotype, complicating development and increasing cost. While CRISPR offers the possibility of precise correction, its practical utility in a genetically diverse PKU cohort remains limited without the development of universal editing approaches.

The rAAV-based gene therapy is the most advanced platform, with multiple clinical trials for monogenic liver disorders providing a regulatory and manufacturing roadmap. However, no PKU-specific rAAV trial has yet been initiated. Naked DNA vectors remain firmly in the preclinical domain due to unsolved delivery barriers. CRISPR/Cas9 is in early-stage development for PKU, with no human trials to date. Significant advances in vector design, editing efficiency, and safety validation are required before clinical translation can be contemplated.

Collectively, the evidence positions rAAV as the most immediately translatable approach for PKU gene therapy, given its strong efficacy and established clinical track record. However, CRISPR/Cas9 holds unparalleled curative potential by enabling permanent genetic correction at the native PAH locus. Future research must prioritize hybrid strategies—such as AAV-delivered CRISPR systems for targeted PAH integration—that combine the delivery efficiency of viral vectors with the durability of genome editing. Additionally, investments in non-viral delivery platforms, improved editing fidelity, and immunomodulatory regimens will be essential to advance these therapies toward clinical reality.

IV. Conclusion

This study aimed to identify the most promising gene therapy approach for Phenylketonuria (PKU) through a comparative analysis of preclinical experiments involving rAAV vectors, naked DNA vectors, and CRISPR/Cas9 gene editing. Findings reveal distinct strengths and limitations across each modality. The rAAV vectors, particularly liver-targeted serotypes such as rAAV2/8 and scAAV2/8, achieved robust and durable Phe correction in mice but were constrained by dose-dependent efficacy and immunogenic risks. Naked DNA vectors, while safe and effective in murine models, face insurmountable translational barriers due to inefficient delivery methods that are not scalable to humans. In contrast, CRISPR/Cas9 emerged as the most promising candidate for a curative therapy due to its ability to correct the underlying PAH mutation directly and permanently, though its current utility is limited by incomplete editing, efficiency and unresolved safety concerns, such as off-target effects. Key limitations of this analysis include its reliance on murine models, which may not fully recapitulate human PKU pathophysiology or immune responses, and the absence of long-term safety data from large-animal studies. Furthermore, the heterogeneity of PAH mutations presents a persistent challenge for mutation-specific approaches like base editing, whereas gene replacement strategies remain vulnerable to vector-related immunogenicity. Future research must prioritize the development of safer, more efficient delivery systems for CRISPR/Cas9 components, rigorous assessment of off-target editing in relevant preclinical models, and exploration of combination approaches that may enhance durability and broaden applicability across PKU genotypes. Ultimately, with continued innovation, gene editing holds transformative potential to deliver the first permanent treatment for PKU, liberating patients from lifelong dietary restrictions and transient pharmacological interventions.

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