Precision Phage Cocktails to Deplete Urease-Producing Gut Bacteria and Lower Ammonia in Hepatic Encephalopathy

Project Summary / Abstract

Hepatic encephalopathy (HE) is a disabling, recurrent neuropsychiatric complication of cirrhosis driven largely by gut-derived ammonia. Urease-producing gut bacteria hydrolyze urea to ammonia, which a failing liver cannot clear; the resulting hyperammonemia crosses the blood–brain barrier and produces confusion, asterixis, and coma. Current mainstays — lactulose and the poorly absorbed antibiotic rifaximin — act non-specifically, broadly perturb the microbiome, and are constrained by tolerability and resistance concerns. Bacteriophages offer a mechanistically distinct strategy: a rationally assembled lytic cocktail can deplete specific urease-rich strains through strain-specific surface receptors while sparing ammonia-neutral commensals, shrinking the community's capacity to convert urea to ammonia at its luminal source.

This proposal builds directly on two anchoring findings. Bajaj et al. (*Gut* 2021) showed, in cirrhotic patients, that gut phage–bacterial linkages centered on urease-producing, ammonia-generating *Streptococcus* species, shifted with disease progression, and collapsed after rifaximin — establishing that phages already co-regulate the precise flora implicated in HE. Duan et al. (*Nature* 2019) demonstrated that an orally delivered, strain-specific phage cocktail against cytolysin-producing *Enterococcus faecalis* abolished ethanol-induced liver disease in humanized mice, whereas phages against non-cytolytic strains did not — direct proof that strain-specific phages can edit a gut pathobiont and improve liver injury.

We will (Aim 1) build a genome-characterized bank of urease-positive gut strains and matched lytic phages from cirrhotic patients; (Aim 2) assemble and optimize a multi-phage cocktail and test ammonia lowering, host-range breadth, and resistance suppression in vitro and in colonized rodent models, against pre-specified quantitative milestones; and (Aim 3) generate the safety, biomarker-stratification, and manufacturing-quality package needed to enable future first-in-human evaluation. The work is explicitly pre-clinical-to-translational and targets a disease for which no registered human phage trial yet exists, aiming to yield the first strain-targeted approach to lowering blood ammonia in advanced liver disease.

Specific Aims

Hepatic encephalopathy reflects gut-derived ammonia that a cirrhotic liver cannot clear. The ammonia-generating trait is carried by identifiable urease-producing taxa, making them rational targets for phage-mediated, strain-level depletion that spares protective commensals. Bajaj et al. (*Gut* 2021) localized the relevant cirrhosis phage–bacterial linkages to urease-producing *Streptococcus*; we extend this to a broader set of urease-positive pathobionts (*Streptococcus*, *Klebsiella*, *Proteus*, *Enterococcus*) defined empirically in our own cohort rather than assumed.

Aim 1. Build a characterized bank of urease-positive gut bacterial strains and matched lytic phages from cirrhotic patients. We will isolate and genome-characterize urease-positive *Streptococcus*, *Klebsiella*, *Proteus*, and *Enterococcus* from cirrhosis/HE stool, recover lytic (strictly virulent) phages against them, confirm urease (and, where relevant, cytolysin) genes in target strains, and map host ranges. *Success criterion:* a verified panel of ≥[ILLUSTRATIVE] 20 urease-positive strains and ≥[ILLUSTRATIVE] 30 sequenced lytic phages with a complete host-range matrix.

Aim 2. Assemble and optimize a phage cocktail and test ammonia lowering in vitro and in vivo. We will combine phages with complementary, distinct-receptor host ranges to broaden coverage and suppress resistance; quantify reductions in urease activity and ammonia in single-strain and defined-community culture; and evaluate oral cocktail delivery in gnotobiotic/humanized rodents colonized with patient urease-positive strains, following the strain-specific paradigm of Duan et al. (*Nature* 2019). *Go/no-go criteria:* ≥[ILLUSTRATIVE] 2-log reduction in target-strain burden, ≥[ILLUSTRATIVE] 30% reduction in blood ammonia versus vehicle (pre-registered, sex-balanced, blinded), and no significant loss of off-target commensal diversity.

Aim 3. Generate the safety, biomarker-stratification, and manufacturing-quality package to enable first-in-human evaluation. We will define purity/endotoxin specifications, perform rodent biodistribution and tolerability studies of the lead cocktail, and develop stratification logic linking gut phage/bacterial signatures to HE (per Peña Rodríguez et al., 2024) to identify likely responders. *Deliverable:* an endotoxin-qualified lead cocktail, a tolerability/biodistribution dossier, and a candidate responder-stratification biomarker set positioned for a future investigator-initiated regulatory (eIND/IRB) submission.

Impact: If successful, this work would deliver the first rationally designed, strain-targeted phage cocktail to lower gut-derived ammonia in cirrhosis — potentially reducing HE without the non-specific microbiome suppression of current therapy.

Significance

HE is among the most burdensome complications of cirrhosis, driving recurrent hospitalizations, caregiver strain, and loss of independence [ILLUSTRATIVE: insert epidemiologic burden statistics finalized to an allowable source]. Its proximate driver is gut-derived ammonia: urease-producing bacteria convert urea to ammonia, and a failing liver cannot detoxify the load, allowing neurotoxic ammonia to reach the brain. Lactulose and rifaximin remain standard but act non-specifically — lactulose through osmotic catharsis and luminal acidification (limited by diarrhea and adherence), rifaximin through broad luminal antibacterial suppression (raising tolerability and resistance concerns). Neither selectively removes the urease-bearing taxa responsible for ammonia generation, leaving a clear mechanistic gap.

Three lines of evidence make a strain-targeted approach compelling. First, Bajaj et al. (*Gut* 2021) sequenced the bacterial metagenome and virome of cirrhotic patients and found that phage–bacterial linkages centered on urease-producing, ammonia-generating *Streptococcus* species shifted with disease progression and collapsed after rifaximin — establishing that phages already co-regulate the precise flora implicated in HE. Second, Duan et al. (*Nature* 2019) showed that an orally delivered phage cocktail specific to cytolysin-producing *E. faecalis* abolished alcohol-associated liver disease in humanized mice, whereas phages against non-cytolytic strains did not — direct proof that strain-specific phages can edit a gut pathobiont and improve liver injury. Third, Peña Rodríguez et al. (2024) tied distinct gut phage and viral-like-particle signatures to complications of cirrhosis, supporting biomarker-guided stratification.

The therapeutic node is independently validated: gut-bacterial urease inhibition with 2-octynohydroxamic acid lowered blood ammonia in rodent models of liver injury (Evstafeva et al., *Nat Commun* 2024), confirming that reducing urease-driven ammonia production is a tractable, disease-relevant lever. Phage cocktails attack the same node not by pharmacologically inhibiting the enzyme but by depleting the bacteria that carry it — a complementary, self-amplifying strategy. Success would advance NIDDK's liver-disease mission and is also relevant to the alcohol-associated cirrhosis population.

Innovation

This proposal is innovative in three principal respects.

1. **Mechanism — deplete the trait-bearing bacteria, not the enzyme or the whole community.** Rather than inhibiting urease pharmacologically or broadly suppressing the microbiome, the cocktail depletes the specific urease-carrying strains while preserving ammonia-neutral commensals, directly addressing the selectivity gap left by lactulose and

rifaximin.

2. **Cocktail design for coverage and durability.** Combining phages with complementary, distinct-receptor host ranges broadens coverage across the heterogeneous urease-producing flora of cirrhosis and suppresses resistance; depolymerase-bearing phages can degrade the capsular polysaccharide and biofilm matrix that protect encapsulated pathobionts such as *Klebsiella*. Orally delivered lytic phages self-amplify with each round of bacterial killing, so a small dose acts locally without systemic antibiotic exposure.

3. **Patient-matched logic with a defined engineering horizon.** Stool sequencing can identify a patient's urease-rich taxa to match a cocktail, and rational pairing with rifaximin offers potential additive microbiome editing analogous to phage–antibiotic synergy. Engineered/CRISPR-armed phages provide a future route to sequence-specific targeting of the urease operon, positioning this program beyond the immediate award.

Approach

Aim 1 — Strain and phage discovery against urease-producing gut flora

Rationale. A precision cocktail requires a well-characterized panel of the actual ammonia-generating strains and the lytic phages that kill them. Bajaj et al. (*Gut* 2021) localized the relevant phage–bacterial linkages to urease-producing *Streptococcus*, providing a defined anchor target; other urease-positive pathobionts (*Klebsiella*, *Proteus*, *Enterococcus*) are included on first principles and resolved empirically in our cohort rather than assumed from that study.

Experimental design. From banked and prospectively collected cirrhosis/HE stool (Team & Environment), we will culture and isolate urease-positive *Streptococcus*, *Klebsiella*, *Proteus*, and *Enterococcus*, confirming urease positivity biochemically and by whole-genome sequencing for urease operons (and cytolysin genes where applicable). Lytic phages will be recovered from patient samples and environmental sources against these strains, plaque-purified, and sequenced to confirm a strictly virulent (non-temperate) lifestyle and the absence of known toxin, antibiotic-resistance, and integrase genes. Host ranges will be mapped across the full strain panel by quantitative efficiency-of-plating.

Rigor & reproducibility. Strains and phages will be archived in duplicate with verified provenance; all genomes deposited; host-range assays performed in biological triplicate with defined acceptance thresholds. Donor sex and clinical stage will be recorded for downstream balancing.

Expected outcomes. A genome-characterized biobank of ≥[ILLUSTRATIVE] 20 urease-positive

target strains with ≥[ILLUSTRATIVE] 30 matched lytic phages and a host-range matrix distinguishing broad- from narrow-range candidates.

Potential pitfalls & alternatives. Some urease-positive taxa may be fastidious or yield few phages. We will broaden sampling, enrich phages from wastewater, and prioritize the most abundant/expandable urease clades; depolymerase-bearing phages will be prioritized for encapsulated *Klebsiella*. If patient-derived phages are scarce for a target, we will source type-strain phages and confirm activity against patient isolates.

Aim 2 — Cocktail assembly and ammonia-lowering efficacy in vitro and in vivo

Rationale. Duan et al. (*Nature* 2019) showed strain specificity is decisive — phages matched to the pathogenic strain worked while mismatched phages did not. A cocktail must therefore be assembled empirically for coverage, resistance suppression, and functional ammonia lowering, not phage count alone.

Experimental design. Using the Aim 1 matrix, we will compose candidate cocktails maximizing host-range coverage with complementary receptor usage. *In vitro:* quantify killing, urease activity, and ammonia production in single-strain and defined-community cultures, and measure resistance emergence across serial passage, including rifaximin pairing for additive suppression. *In vivo:* gnotobiotic/humanized rodents colonized with patient urease-positive strains will receive oral cocktail versus vehicle; primary readouts are blood ammonia and target-strain burden, with 16S/shotgun metagenomic profiling to confirm sparing of off-target commensals.

Rigor & reproducibility. In vivo studies will be randomized, blinded for outcome assessment, powered by explicit a priori analysis, and conducted in both sexes (sex as a biological variable). Cohorts will be replicated across independent colonization batches.

Go/no-go criteria. A lead cocktail advances if it achieves ≥[ILLUSTRATIVE] 2-log reduction in target-strain burden, ≥[ILLUSTRATIVE] 30% reduction in blood ammonia versus vehicle, and no significant reduction in off-target commensal diversity. Cocktails failing the ammonia threshold despite burden reduction trigger reformulation toward higher-urease clades.

Expected outcomes. A lead cocktail that reduces target-strain burden and lowers ammonia in vivo while minimally perturbing the broader community, with characterized resistance-suppression behavior.

Potential pitfalls & alternatives. Bacteria may evolve phage resistance or receptor loss; we will counter with multi-receptor cocktails, depolymerase-armed phages, and rifaximin pairing. If the ammonia signal is modest in one model, we will use alternative colonization/diet-stress paradigms

consistent with the humanized-mouse approach validated by Duan et al.

Aim 3 — Safety, biomarker stratification, and manufacturing-quality package

Rationale. Because no registered human phage trial for HE exists, a credible path to eventual first-in-human use requires safety, stratification, and manufacturing-quality groundwork generated in advance.

Experimental design. We will define purity/endotoxin specifications and run rodent biodistribution and tolerability studies for the lead cocktail. We will develop stratification logic linking gut phage/bacterial signatures to HE and outcomes, per Peña Rodríguez et al. (2024), to identify likely responders. We will assemble the manufacturing-quality and safety documentation needed to support a future investigator-initiated regulatory submission (eIND with IRB oversight) for compassionate use in refractory patients — preparation, not execution, within this award.

Rigor & reproducibility. Biodistribution and tolerability assays will use validated, quantitative methods with predefined endpoints; biomarker analyses will be pre-specified and treated as exploratory/hypothesis-generating, with correction for multiple comparisons.

Expected outcomes. An endotoxin-qualified lead cocktail, a tolerability/biodistribution dossier, a candidate responder-stratification biomarker set, and assembled documentation positioned for a future eIND/IRB submission.

Potential pitfalls & alternatives. Endotoxin or manufacturing hurdles may arise with Gram-negative-targeting phages; we will optimize purification and, if needed, prioritize the lowest-endotoxin lead. Biomarker–outcome links may be weaker than reported; stratification will remain exploratory and hypothesis-generating for a future trial.

Timeline

[ILLUSTRATIVE] **Years 1–2:** Aim 1 strain/phage isolation, sequencing, and host-range mapping. **Years 2–3:** Aim 2 in vitro cocktail optimization and initial in vivo studies; first go/no-go decision. **Years 3–4:** Aim 2 confirmatory in vivo efficacy; begin Aim 3 safety/biodistribution. **Years 4–5:** Aim 3 endotoxin qualification, stratification analysis, and regulatory-package assembly.

Budget Justification (modular R01-style sketch)

[ILLUSTRATIVE] Request: [ILLUSTRATIVE] $250,000 direct costs/year for [ILLUSTRATIVE] 5 years. **Personnel:** PI ([ILLUSTRATIVE] 2.4 calendar months), co-investigators in hepatology and

phage biology, [ILLUSTRATIVE] 2 postdocs, and a research technician for strain/phage culture and animal work. **Supplies:** microbiology/phage isolation, whole-genome sequencing, ammonia/urease assays, and gnotobiotic rodent husbandry. **Other:** metagenomic/virome sequencing core fees, endotoxin testing, and regulatory consulting for eIND preparation. Modular budget and headcounts are illustrative placeholders to be finalized with institutional rates.

Vertebrate Animals

Animal work is proposed (Aim 2 efficacy; Aim 3 biodistribution/tolerability). Gnotobiotic/humanized rodents will be colonized with patient-derived urease-positive strains and given oral phage cocktail or vehicle, following the orally delivered, strain-specific paradigm of Duan et al. (*Nature* 2019). Endpoints include blood ammonia, target-strain burden, microbiome profiling, and tolerability. Studies will be randomized, blinded for outcome assessment, and conducted in both sexes. Group sizes ([ILLUSTRATIVE]) will be set by a priori power analysis; humane endpoints, anesthesia/euthanasia, and IACUC approval will follow institutional and federal policy, with the 3Rs applied to minimize animal number and distress.

Human Subjects / Clinical Trial

No interventional clinical trial is proposed within this R01. Human involvement is limited to (a) collection/use of cirrhosis/HE stool and clinical metadata for strain/phage discovery and biomarker stratification (Aims 1, 3), under IRB approval with informed consent, and (b) preparation — not execution — of a regulatory pathway for future compassionate use. Any first-in-human administration of the investigational phage cocktail would proceed only under an FDA emergency/expanded-access IND (eIND) with IRB oversight, applied per patient for refractory HE, and is positioned here as a downstream step pending the safety package developed in Aim 3.

Team & Environment

[PI — Name, Institution]: hepatology/microbiome therapeutics lead with cirrhosis virome expertise (template role aligned to the Bajaj group, Virginia Commonwealth University / Richmond VA Medical Center). **[Co-I — Name, Institution]:** phage targeting of gut pathobionts in liver disease (template role aligned to the Schnabl lab, UC San Diego). **[Co-I — Name, Institution]:** gut metagenome/virome analysis (template role aligned to the Gillevet/Microbiome Analysis Center, George Mason University). **[Co-I — Name, Institution]:** translational phage manufacturing/regulatory support (template role aligned to an academic phage center such as the Center for Innovative Phage Applications and Therapeutics, UC San Diego). **Environment:**

institutional gnotobiotic rodent facilities, sequencing/virome cores, BSL-appropriate phage labs, and an academic medical center caring for cirrhosis patients. Names and institutions to be finalized.

References

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2. Duan Y, Llorente C, Lang S, et al. Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. *Nature.* 2019 Nov;575(7783):505-511. https://doi.org/10.1038/s41586-019-1742-x

3. Peña Rodríguez M, Fagan A, Sikaroodi M, Gillevet PM, Bajaj JS. Proton Pump Inhibitor Use and Complications of Cirrhosis Are Linked With Distinct Gut Microbial Bacteriophage and Eukaryotic Viral-Like Particle Signatures in Cirrhosis. *Clin Transl Gastroenterol.* 2024 Feb 1;15(2):e00659. https://pubmed.ncbi.nlm.nih.gov/37937851/

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