Gut Microbiome-Derived Uremic Toxin Levels in Hemodialysis Patients on Different Phosphate Binder Therapies

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Keywords
Hemodialysis · Uremic toxins · Phosphate binders · Sucroferric oxyhydroxide · Sevelamer carbonate

Abstract
Introduction: Constipation is prevalent in patients with kidney failure partly due to the use of medication, such as phosphate binders. We hypothesized that serum levels of gut microbiome-derived uremic toxins (UTOX) may be affected by the choice of phosphate binder putatively through its impact on colonic transit time. We investigated two commonly prescribed phosphate binders, sevelamer carbonate (SEV) and sucroferric oxyhydroxide (SFO), and their association with gut microbiome-derived UTOX levels in hemodialysis (HD) patients. Methods: Weekly blood samples were collected from 16 anuric HD participants during the 5-week observational period. All participants were on active phosphate binder monotherapy with either SFO or SEV for at least 4 weeks prior to enrollment. Eight UTOX (7 gut microbiome-derived) and tryptophan were quantified using liquid chromatography-mass spectrometry. Serum phosphorus, nutritional, and liver function markers were also measured. For each substance, weekly individual levels, the median concentration per participant, and differences between SFO and SEV groups were reported. Patient-reported bowel movements, by the Bristol Stool Scale (BSS), and pill usage were assessed weekly. Results: The SEV group reported a 3.3-fold higher frequency of BSS stool types 1 and 2 (more likely constipated, \( p < 0.05 \)), whereas the SFO group reported a 1.5-fold higher frequency of BSS stool types 5–7 (more likely loose stool and diarrhea, not significant). Participants in the SFO group showed a trend toward better adherence to phosphate binder therapy (SFO: 87.6\% vs. SEV: 66.6\%, not significant). UTOX, serum phosphorus, nutritional and liver function markers, and tryptophan were not different between the two groups. Conclusion: There was no difference in the gut microbiome-derived UTOX levels between phosphate binders (SFO vs. SEV), despite SFO therapy resulting in fewer constipated participants. This pilot study may inform study design of future clinical trials and highlights the importance of including factors beyond bowel habits and their association with UTOX levels.

Introduction
The loss of kidney function in kidney failure patients is accompanied by the retention of a host of metabolites [1]. Many of these uremic retention solutes have been shown to negatively affect the functioning of cells and or-
gans, resulting in the uremic syndrome [2]. Some of these uremic toxins (UTOX) are derived from colon microbes [3–7]. Gut microbiome dysbiosis – changes in composition and function of the gut microbiome – is common in kidney failure patients. One contributing factor to gut dysbiosis is the change of gastrointestinal tract environment by the influx of urea, uric acid, and oxalate from the blood into the gut. A previous study revealed that the dominant microbiota in kidney failure patients are families expressing urease, urate oxidase, and p-cresol- and indole-forming enzymes [8]. These enzymes generate metabolites that are subsequently absorbed into the blood and, in some cases, further metabolized into UTOX in the liver. Some toxins are highly protein-bound and thus are inefficiently removed by conventional hemodialysis (HD) treatment [9]. Their accumulation in kidney failure patients may contribute to systemic inflammation and chronic kidney disease (CKD) progression [10–12]. Further, elevated levels of these protein-bound UTOX have been associated with higher all-cause and cardiovascular mortality [13]. However, it is important to acknowledge that to date no causal relationship between colon-derived UTOX and poor clinical outcomes has been established.

Dialysis patients frequently suffer from constipation as a result of prolonged colonic transit time [14]. Prolonged colonic transit time can result in increased protein fermentation, altered gut microbiota, and subsequent changes in the production of UTOX precursors [7]. Chronic constipation is partly due to the use of medication, such as phosphate binders [14]. Interestingly, one of the significant side effects of iron-based (sucroferric oxyhydroxide [SFO]) phosphate binders is more frequent bowel movements, loose stool, and diarrhea [15–17]. If patients on SFO (Velphoro®) experience more frequent bowel movements, their colonic transit time may be shorter than patients on a widely prescribed phosphate binder, sevelamer carbonate (SEV; Renvela®), who reported higher rates of constipation [15]. The combination of these factors may induce overgrowth of proteolytic species, which favors the production of both toxic metabolites (e.g., p-cresyl sulfate [PCS], phenyl acetic acid, indoxyl sulfate [IS], and indole 3-acetic acid) and proinflammatory substances (e.g., PCS) in kidney failure patients [18, 19].

We hypothesized that serum levels of gut microbiome-derived UTOX may be affected by the choice of phosphate binder putatively through its impact on colonic transit time. This hypothesis was built on two observations: (a) clinically, SEV induces constipation, while SFO causes transient diarrhea [15]; (b) colonic transit time impacts the generation of gut-derived UTOX as longer colonic transit time is associated with higher UTOX levels [7].

Materials and Methods

Study Design

The study presented here is a multicenter, 6-week observational study conducted at 4 HD clinics in New York, NY, USA. The inclusion criteria were kidney failure patients on thrice weekly HD regimen, ≥18 years of age at the time of informed consent, and on active phosphate binder monotherapy with either SFO or SEV for at least 4 weeks prior to enrollment. Seventeen study participants were enrolled from September 2018 to March 2019 with no follow-up afterward. The exclusion criterion was inability to maintain dietary to track binder usage and bowel movements. Sixteen study participants completed all study visits; 1 patient was withdrawn due to relocation (Fig. 1). All study participants were anuric and treated with polysulfone dialyzers.

Study participants were seen on a scheduled mid-week treatment day once a week for 6 consecutive weeks. An interview was administered in the first week, which included study participants’ binder prescription and bowel habits. In the subsequent 5 weeks, predialysis blood samples were collected to measure UTOX and other biomarkers related to phosphate binder therapy, nutritional status, and liver function. Seven gut-derived UTOX were selected to test the hypothesis: trimethylamine-N-oxide (TMAO), indoxyl glucuronide (IG), phenylacetylglutamine (PAG), IS, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), PCS, and hippuric acid (HA; derived from both the gut microbiome and mammalian sources). For comparison, we also measured mammalian-derived kynurenic acid (KYNA) and nutritional marker tryptophan (TRP). A weekly diary was provided to the study participants to capture phosphate binder usage and bowel movements. Satisfaction and frequency of bowel movements along with stool consistency, determined by Bristol Stool Scale (BSS) [20], were assessed to indicate the bowel habit for each patient. On the BSS, types 1 and 2 were considered as likely constipated, types 5–7 as likely loose stool or diarrhea, and types 3 and 4 were regarded as normal. Bowel satisfaction was evaluated using a numeric scale from 1 (unsatisfactory) to 10 (completely satisfactory).

The adherence to phosphate binder therapy was determined by weekly pill counts. The adherence was calculated as the percentage of the actual number (pill count) to the expected number (prescription) of pills consumed. UTOX levels, phosphate binder usage and bowel habits, were analyzed until March 2021. All study procedures were approved by Western Institutional Review Board® (Protocol number: #20181972) and performed in accordance with the principles of the Declaration of Helsinki. The signed written informed consent forms were obtained from all participants for this study.

Sample Preparation

Serum was aliquoted and stored at −80°C until analysis. For UTOX and TRP analysis, samples were prepared according to Dunn et al. [21] with some modifications. Briefly, 50 µL serum was spiked with 25 µL internal standard cocktail consisting of [D₆]-trimethylamine N-oxide, [¹³C₆]-IS, and [D₆]-PCS (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). Aliquots of serum from each participant were combined and served as quality control.
samples. An external standard curve for each substance of interest quantified by liquid chromatography–mass spectrometry (LC-MS) was prepared in 4% human serum albumin. Samples were extracted with 150 µL ice-cold methanol followed by centrifugation for 15 min at 4,300 rpm and 4°C. The supernatant was dried down using a centrifugal vacuum evaporator without heating and dried extracts were resuspended in 0.01% formic acid and 1 mM ammonium formate for LC-MS analysis.

Sample Analysis
Serum phosphate, nutritional markers, and liver enzymes were analyzed using a Pentra C400 biochemistry analyzer (Horiba, Irvine, CA). UTOX and TRP levels were analyzed using a 1290 Infinity II high-performance liquid chromatograph coupled to a 6545 Quadrupole Time-of-Flight mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). UTOX and TRP were separated on a reverse-phase column (Luna C18 4.6 × 150 mm, 3 µm, Phenomenex, Torrance, CA, USA) at a flow rate of 0.75 mL/min. The gradient mobile phase consisted of mobile phase A (0.01% formic acid and 1 mM ammonium formate in water) and mobile phase B (0.01% formic acid and 1 mM ammonium formate in methanol). The gradient started at 1% mobile phase B, which was changed linearly over the next 6.5 min until reaching 40%. Over the next 1.5 min, the percentage was increased linearly to 90% followed by a linear increase to 100% over 3.5 min, where it was kept for 0.5 min. The mobile-phase composition was allowed to return to the initial conditions, which were held for 3 min to equilibrate the column. The following MS parameters were used: m/z 50–1,000, gas temperature 350°C, drying gas 12 L/min, nebulizer 35 psig, sheath gas temperature 400°C, sheath gas flow 12 L/min, fragmentor 125 V and 175 V, skimmer 65 V, Oct 1 RF Vpp 750 V, VCap 4,000 V, and Nozzle Voltage 500 V for positive mode; VCap 2,000 V and Nozzle Voltage 2,000 V for negative mode. A standard curve consisting of 5 levels for UTOX and TRP was used to calculate accuracy and precision for 3 quality control samples measured in triplicates at low, mid, and high concentrations of UTOX and TRP.

Statistical Analysis
Descriptive statistics including measures of central tendency (mean, median) and spread (standard deviation, interquartile range) for continuous variables and number and frequency for categorical variables were compiled. For each UTOX, TRP, and routine clinical diagnostic biomarkers, we calculated the median concentration per participant and then the median across all participants within each group. We also reported the differences in median serum concentrations between the treatment groups. A mixed-effects model was employed to investigate UTOX levels between different binder therapies (SEV vs. SFO) during the observational period (R Foundation for Statistical Computing, Vienna, Austria).

Results
There was no significant difference between the two treatment groups regarding sex, age, race, body mass index, comorbidities, dialysis vintage, adherence to binder therapy, duration of phosphate binder treatment, Kt/V, and normalized protein catabolic rate (Table 1). Pill bur-
den was lower in the SFO group versus the SEV group (mean 2.6 vs. 6.4, respectively). Simultaneous lanthanum carbonate intake was recorded for 1 patient in the SEV group. The SEV group reported a 3.3-fold higher frequency of stool types 1 and 2 (more likely constipated, \( p < 0.05 \)), whereas the SFO group reported a trend toward a 1.5-fold higher frequency of stool types 5–7 (more likely loose stool and diarrhea, not significant) (Fig. 2). When scoring bowel habit satisfaction on a scale from 1 (unsatisfactory) to 10 (completely satisfactory), patients in the SFO group showed a trend toward higher scores than the SEV group (7.1 vs. 5.5, respectively). Individual phosphate levels, nutritional markers, such as normalized protein catabolic rate, albumin and total protein, liver function markers, and total and direct bilirubin were assessed over 5 weeks (Fig. 3; Table 1). Phosphate levels did not differ between the SEV and SFO groups (Table 2). A total

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**Table 1. Study participants’ characteristics**

| Characteristic                                    | Total (\( n = 16 \)) | SEV (\( n = 8 \)) | SFO (\( n = 8 \)) |
|--------------------------------------------------|-----------------------|-------------------|-------------------|
| Female/male                                       | 5/11                  | 3/5               | 2/6               |
| Age, years                                        | 55.1±13.7             | 58.3±13.5         | 51.9±14.0         |
| BMI, kg/m²                                        | 27.5±5.9              | 27.1±7.1          | 27.9±4.9          |
| Hemodialysis vintage, years                       | 4.8±3.8               | 5.1±4.6           | 4.4±3.2           |
| Equilibrated Kt/V                                 | 1.38±0.16             | 1.42±0.20         | 1.35±0.12         |
| Duration of phosphate binder treatment, years     | 2.0±1.6               | 3.0±1.9           | 1.3±0.8           |
| Phosphate binder treatment adherence,* %          | 77.1±34.5             | 66.6±25.0         | 87.6±40.9         |
| Bowel habit satisfaction score**                  | 6.3±2.6               | 5.5±3.5           | 7.1±1.5           |
| nPCR, g/kg/day                                    | 1.03±0.17             | 1.05±0.22         | 1.01±0.12         |
| Race, n (%)                                       |                       |                   |                   |
| White                                            | 8 (50)                | 5 (50)            | 4 (50)            |
| African-American                                  | 7 (44)                | 4 (50)            | 3 (37)            |
| Asian                                            | 1 (6)                 | 0 (0)             | 1 (13)            |
| Comorbidities, n (%)                              |                       |                   |                   |
| Type 2 diabetes                                   | 3 (19)                | 1 (13)            | 2 (25)            |
| Hypertension                                     | 13 (81)               | 6 (75)            | 7 (88)            |
| Chronic heart failure                             | 1 (6)                 | 1 (13)            | 0 (0)             |
| Secondary hyperparathyroidism                     | 16 (100)              | 8 (100)           | 8 (100)           |
| HIV                                              | 3 (19)                | 1 (13)            | 2 (25)            |

Values are mean±standard deviation or count (percentage) as appropriate. BMI, body mass index; SEV, sevelamer carbonate; SFO, sucroferric oxyhydroxide; nPCR, normalized protein catabolic rate; HIV, human immunodeficiency virus.

* The phosphate binder treatment adherence was calculated as the percentage of the actual number (pill count) to the expected number (prescription) of pills consumed.

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\text{Adherence} = \frac{\text{pill count}}{\text{prescription (pill number expected to be taken)}} \times 100\%.
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**Bowel habit satisfaction on a scale of 1–10 was assessed at the time of enrollment and reported by the study participants.**

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**Fig. 2.** Bowel movements in 16 participants over 5 weeks stratified per treatment group. Stool types 1–7 were categorized according to the Bristol Stool Scale. The number 0 is not part of the Bristol Stool Scale. It means an absence of a daily bowel movement. Type 1, nut-like; type 2, lumpy sausage; type 3, sausage with cracks; type 4, smooth snake; type 5, soft blobs; type 6, fluffy pieces; type 7, watery. SFO, sucroferric oxyhydroxide; SEV, sevelamer carbonate.
of 9 substances were measured by LC-MS weekly over 5 weeks for each patient shown in Figure 4: 7 gut microbiome-derived UTOX (TMAO, IG, PAG, IS, CMPF, PCS, and HA), 1 mammalian-derived UTOX (KYNA), and 1 nutritional marker (TRP). UTOX and TRP levels did not differ between the SEV and SFO groups (Table 3; Fig. 4).

**Discussion**

Our study investigated the effect of two widely prescribed phosphate binders, SEV and SFO, on gut microbiome-derived UTOX levels, putatively through its impact on colonic transit time. We discovered SFO therapy
Fig. 4. Serum levels of UTOX and tryptophan in 16 participants over 5 weeks stratified per treatment group. TMAO (a), IG (b), PAG (c), IS (d), CMPF (e), PCS (f), HA (g), KYNA (h), and TRP (i). SFO, sucralfate; SEV, sevelamer carbonate; TMAO, trimethylamine-N-oxide, IG, indoxyl glucuronide; PAG, phenylacetylglutamine; IS, indoxyl sulfate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; PCS, p-cresyl sulfate; HA, hippuric acid; KYNA, kynurenic acid; TRP, tryptophan. Comparison between groups over time was analyzed by a mixed-effects model. The threshold for statistical significance was set to $p < 0.05$. 

Normal range of each compound:
- TMAO: 37.7 ± 20.4 µmol/L ($p = 0.17$)
- IS: 2.5 ± 18.9 µmol/L ($p = 0.37$)
- PAG: 16.8 ± 11.2 µmol/L ($p = 0.83$)
- IS: 1.92 ± 7.5 µmol/L ($p = 0.75$)
- PAG: 10.0 ± 12.4 µmol/L ($p = 0.75$)
- HA: 0.03 ± 0.01 µmol/L ($p = 0.68$)
- KYNA: 67.4 ± 10.2 µmol/L ($p = 0.35$)
resulted in fewer constipated participants, and gut microbiome-derived UTOX levels were not different between the two groups. To the best of our knowledge, we are the first group to compare UTOX levels in HD patients on SFO or SEV in the same setting. Our pilot study could inform future studies, especially for power analysis and sample size estimation.

The bowel habits result from the present study is in agreement with Floege et al. [15], who reported a higher frequency of mild transient diarrhea in patients taking SFO compared to SEV (20% vs. 7%, respectively) and a higher frequency of constipation with SEV compared to SFO (7.2% vs. 3.8%, respectively). In a later phase III, open-label, long-term study to assess safety and efficacy of SFO, the frequency of diarrhea was similarly high at 22.4% [17]. The consistency and frequency of stool could be an indicator of prolonged colonic transit time. Wu et al. [22] reported that total, right segmental as well as rectosigmoid segmental colonic transit times were prolonged in HD patients compared with healthy controls [22]. They further observed that increased individual colonic transit times were correlated with self-reported constipation in HD patients.

The colon is the main site of gut-derived uremic solute production [23]. Prolonged colonic transit time, in addition to the altered composition and function of gut microflora, may result in longer time available for bacterial proliferation [7]. Long colonic transit time is accompanied by a shift in colonic metabolism from carbohydrate fermentation to protein catabolism as reflected by higher urinary levels of potentially deleterious protein-derived metabolites [24]. Delivery of undigested protein to the colon may generate more uremic toxin precursors (such as thiols, phenols, and indoles) through proteolytic fermentations and the toxic waste products are absorbed into systemic circulation [25]. Indeed, studies showed disturbed bowel habits, character-

| Substance | Albumin, g/dL | Total Pt, g/dL | Phosphate, mg/dL | ALP, U/L | ALT, U/L | AST, U/L | B-T, mg/dL | B-D, mg/dL |
|-----------|---------------|---------------|-----------------|--------|--------|--------|---------|---------|
| Normal range | 3.4–5.4 | 6.0–8.3 | 2.5–4.5 | 44–147 | 7–56 | 10–40 | 0.1–1.2 | <0.3 |
| SFO | 3.8 (0.3) | 6.7 (0.4) | 63 (2.0) | 136.3 (77.7) | 14.2 (3.0) | 14.9 (4.0) | 0.4 (0.2) | 0.14 (0.06) |
| SEV | 3.7 (0.2) | 6.6 (0.6) | 6.1 (1.2) | 92.7 (26.5) | 13.4 (15.8) | 18.4 (11.7) | 0.5 (0.2) | 0.15 (0.08) |
| ΔMedian | 0.08 | 0.10 | 0.29 | 43.61 | 0.73 | −3.51 | −0.12 | −0.01 |
| p value | 0.79 | 0.67 | 0.97 | 0.18 | 0.55 | 0.40 | 0.63 | 0.69 |

Values are median (interquartile range [IQR]) serum concentrations in each group. IQR was calculated as the difference between the third and the first quartiles. A mixed-effects model was used for a p value analysis. SFO, sucroferric oxyhydroxide; SEV, sevelamer carbonate; ΔMedian, the median value in the SFO group minus the median value in the SEV group; total Pt, total protein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; B-T, total bilirubin; B-D, direct bilirubin.
ized by slow transit time (constipation), which may lead to production of PCS ($p = 0.02$) in nondiabetic nondialysisdependent CKD patients [26] and higher levels of total and free PCS ($p < 0.01$) and total indole acetic acid ($p = 0.04$) in patients on automated peritoneal dialysis [27]. While the impact of SEV and SFO on colonic transit time is well established [14, 15], we cannot exclude the possibility that inherent differences in the agents themselves may affect solute production or intestinal uptake. Our pilot study strongly suggests that it is crucial for future trials to include detailed information on microbiome composition, colonic metabolism, and dietary habits to understand the causal relationship between colonic transit time and individual uremic toxin levels.

Phosphate binders are also associated with gastrointestinal distress, and they may bind molecules other than phosphate and may alter the gut microbiota, altogether having systemic effects unrelated to phosphate control [28]. The impact of SEV on uremic toxin levels has been controversial. In a large-scale ($N = 423$) observational study by Dai et al. [29] comparing SEV users with nonusers, SEV use was associated with high serum IS. Another observational study found a significant reduction in the levels of PCS, not IS, after a 3-month period of SEV treatment [30]. The precursor of PCS, $p$-cresol, was found to be significantly reduced after SEV treatment in a single-blind, randomized placebo-controlled trial with patients with stage 3–5 CKD (not on dialysis) [31]. In contrast, Brandenburg et al. [32] reported that 8 weeks of sevelamer hydrochloride treatment increased serum PCS but did not change serum IS levels in HD patients. Furthermore, in a multicenter, double-blind, placebo-controlled randomized clinical trial to evaluate the adsorptive effect of SEV in stage 3b–4 CKD patients, there was no significant decrease in the serum concentration of PCS, IS, and indole acetic acid after a 12-week course of treatment [33]. Similarly, in a secondary analysis of the NICOREN trial, SEV did not reduce the circulating levels of protein-bound toxins in HD patients [34].

To date, the knowledge of the impact of SFO on UTOX levels is limited. Only one study explored the impact of SFO on UTOX in HD patients [35] and revealed that, despite a lack of change in the diversity and composition of the microbiome, UTOX (IS and PCS) were increased. It is important to note that the design of this and our study is different. Iguchi et al. studied HD patients before and after 3 months of SFO administration. In the present study, participants were already on SFO binder therapy for at least 4 weeks (on average $1.3 \pm 0.8$ years) and UTOX levels were measured over a shorter period of 5 weeks.

Out of the 9 substances detected by LC-MS in HD serum in the present study, TMAO, IG, PAG, IS, PCS, and HA undergo metabolism in the liver before entering the systemic blood stream. The participants in our study did not show any liver impairment, as three liver markers were not different between the two treatment groups. At the individual level, alkaline phosphatase (ALP) was increased in four SFO participants and one SEV participant. Since ALP exists in blood in numerous distinct forms, which originate mainly from bone and liver, slightly elevated ALP may indicate a disturbance in bone mineral metabolism [36], which is quite common in the kidney failure population. Consistent with previously published literature [37, 38], the two binders SEV and SFO demonstrated equivalent control of serum phosphorus. Additionally, we found that study participants in the SFO group tended to be more compliant to the medication (87.6%) as opposed to patients in the SEV group (66.6%); this may be a result from the lower pill burden of SFO [39].

The limitations of our study are its observational nature and a small sample size, which may mask the impacts of phosphate binder on UTOX blood levels. The evaluations of stool consistency and bowel movements rely primarily on patient-reported information. We observed discrepancies between patient-reported medication consumption and actual pill count. This observation raises a concern about the reliability of patient-reported data and, along with the small sample size, prevents us from conducting further analysis of adjusting confounding factors. Similar to previously published literature by Floege et al. [15], nonadherence was slightly more common in the SEV group than in the SFO group. Evaluation of fecal specimen may give more insightful information with regard to therapy adherence and gut microflora. However, due to logistical difficulties, fecal samples were not obtained during the study. Another drawback of our study is the lack of a detailed dietary assessment that would allow us to capture the protein-to-fiber ratio and the direct intake of UTOX precursors.

In conclusion, there was no difference in the serum gut microbiome-derived UTOX levels between phosphate binders (SFO vs. SEV), although SFO resulted in fewer constipated participants. Future interventional studies with a larger sample size may include factors beyond bowel habits and their role in affecting the UTOX levels.
Phosphate Binders and Uremic Toxin Levels

Conflict of Interest Statement

P.K. holds stock and S.T. holds performance shares in Fresenius Medical Care. The other authors declare no competing interest.

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Author Contributions

L.W. and L.M.T.S. contributed equally to this work. L.W., L.M.T.S., X.T., J.C., O.T., S.T., P.K., and N.G. contributed to the design and implementation of the research. L.W., L.M.T.S., X.T., J.C., H.Z., and N.G. analyzed the data. L.W., L.M.T.S., P.K., and N.G. wrote the manuscript with input from all authors. N.G. directed the project. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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